RNA interference in insects: persistence and uptake of double-stranded RNA and activation of RNAi genes

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A NOTE ON THE STRUCTURE OF THE THESIS

This thesis is structured in a typical manner. A general methods chapter, containing details of the routinely used techniques, is included, although each results chapter contains its own methods section, detailing any procedures specific to the work presented therein. The results are presented as chapters and not as stand-alone papers, except chapter six (*dicer-2* and *argonaute-2* are upregulated in response to dsRNA), which is written as a manuscript for publication. This chapter, consequently, is comparatively brief and contains a degree of repetition.

ABSTRACT

RNA interference (RNAi) is a eukaryotic phenomenon where short double-stranded RNA molecules (dsRNAs) repress homologous sequences. In insects RNAi has been widely observed and has proved extremely useful as a reverse genetics tool to elucidate the function of newly identified genes, as well as showing potential as a novel insecticide. Unfortunately, however, not all insect species are equally susceptible to RNAi. This thesis explores whether persistence of dsRNA in insect hemolymph, uptake of dsRNA into insect tissue, or activation of RNAi genes could be limiting factors in RNAi experiments. Trials were conducted with the tobacco hornworm, *Manduca sexta*, a species in which experimental difficulty has been experienced with RNAi protocols and the German cockroach, *Blattella germanica*, which is known to be highly susceptible to experimental RNAi. In *M. sexta* larvae dsRNA disappeared rapidly from the hemolymph *in vivo*. By comparison, exogenous dsRNA persisted longer in the hemolymph of *B. germanica* adults. These findings lead me to propose that the rate of persistence of dsRNA in insect hemolymph may be a key factor in determining the susceptibility of insect species to RNAi. Despite such rapid breakdown of dsRNA in *M. sexta* larvae uptake of exogenous dsRNA into hemocytes, fat body and midgut could be detected by quantitative RT-PCR *in vivo* and was experimentally investigated in hemocytes *in vivo* and *in vitro* using fluorescently labelled dsRNA. Furthermore, quantitative-RT-PCR revealed that the expression of two *M. sexta* RNAi genes *dicer-2* and *argonaute-2* (partial sequences of which were isolated during this study) was specifically upregulated in response to injection with dsRNA.

ABBREVIATIONS

A – Adenine

Ago-1 – Argonaute-1

Ago-2 – Argonaute-2

Ago-3 – Argonaute-3

al-1 - albino-1

al-3 - albino-3

AMP – Antimicrobial Peptide

°C – degrees Celsius

% - Percent

BLAST - Basic Local Alignment Search Tool

bp - base pair

C – Cytosine

CFU - Colony Forming Units

CHS - chalcone synthase

C_T – Baseline-subtracted Threshold

cDNA – complementary DNA

C3PO - Component 3 Promoter Of RISC

DEPC - diethylpyrocarbonate

Dfd - deformed

DNA - deoxyribonucleic acid

dNTP - deoxyriboNucleotide TriPhosphate

dsRNA – double-stranded RNA

esiRNA - endogenous siRNA

EST – Expressed Sequence Tag

EtBr – Ethidium Bromide

eve - even-skipped

FITC – Fluorescecein Isothiocyanate

FSC-H – Forward Scatter

ftz - fushi tarazu

G – Maximum Relative Centrifugal Force or G-Force

g – grams

G – Guanine

GFP - Green Fluorescent Protein

GI – Genbank ID

GIM - Grace's Insect Medium

h – hour

Hox - Homeobox

IMD – Immune Deficient

IPTG - Isopropyl β-D-1-thiogalactopyranoside

kDa – KiloDalton

LB – Luria Bertani

M – Molar

MAMP – Microbial-Associated Molecular Pattern

MAP4K4 - mitogen-activated protein kinase kinase kinase kinase

mg - milligrams

ml – millilitres

mM – millimolar

mRNA - messenger RNA

µg – micrograms

miRNA – micro RNA

µL – microlitres

µM – micromolar

ng - nanogram

nt – nucleotide

PAR-1 - partition 1

pb - proboscipedia

PBS – Phosphate Buffered Saline

PCR - Polymerase Chain Reaction

PFA - Paraformaldehyde

piRNA - piwi-interacting short RNA

Piwi - P-element induced wimpy testis

*p*mol – picomole

PRR – Pattern Recognition Receptor

PTU - phenylthiourea

q-RT-PCR – quantitative Reverse Transcription PCR

RACE - Rapid Amplification of cDNA Ends

RdRp - RNA-dependent RNA polymerase

RISC - RNA-induced silencing complex

RLC - RISC-loading complex

RNA – ribonucleic acid

RNAi – RNA interference

rpm – revolutions per minute

RSV - Respiratory Syncytial Virus

RT - Reverse Transcription

Scr - Sex combs reduced

SDS-PAGE – Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

shRNA – small hairpin RNA

sid-1 - systemic RNA interference deficient-1

siRNA – short interfering RNA

SSC-A – Side Scatter

ssRNA – Single-Stranded RNA

T - Thymine

TAE – Tris Acetate EDTA

TE - Transposable Element

TEM – Transmission Electron Microscope

Tm – Melting Temperature

TRAX - translin-associated factor-X

TSN - Tudor-staphyloccal nuclease

U – units

VIG - vasa intronic gene

1. INTRODUCTION

1.1 Introduction to RNA interference

1.1.1 RNA interference is a gene silencing phenomenon

RNA interference (RNAi) is a term used to describe a number of gene silencing phenomena characterised by the specific binding of short RNAs (20-30 nucleotides in length) to target sequences in a process mediated by Argonaute family proteins (Zaratiegui *et al.*, 2007; Obbard *et al.*, 2009). In this dissertation, the term RNAi is predominantly used to describe one specific type of RNAi, exogenous RNAi, where short RNAs of exogenous origin bind specifically to endogenous target RNA sequences leading to cleavage of the targeted endogenous RNA (Figure 1.1). Exogenous RNAi is evolutionarily conserved in eukaryotes, although it has been lost from some lineages, and has proved to be an invaluable tool for the characterisation of newly identified genes in eukaryotic organisms.

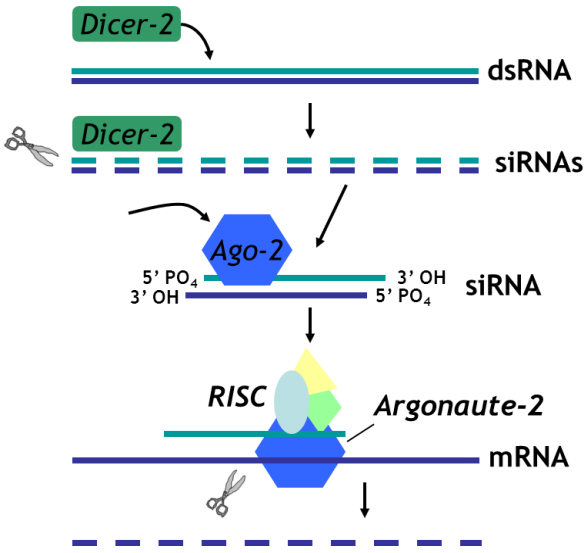


Figure 1.1: Exogenous RNA interference (RNAi) in (for example) *Drosophila melanogaster*. Double-stranded RNAs (dsRNAs) are processed by the enzyme Dicer-2, being cleaved into short RNA duplexes 21-23 nucleotides in length termed "short interfering RNAs" (siRNAs). One siRNA strand is released whilst the remaining strand binds to its complementary messenger RNA (mRNA). The RISC (RNA-induced silencing complex) component Argonaute-2 (Ago2) cleaves the mRNA, targeting it for degradation.

1.1.2 A short history of the discovery of RNAi

Gene silencing mediated by exogenous dsRNA (i.e. classical RNA interference, in the sense that the term is used here) was first described in 1998 by Andrew Fire and Craig Mello (Fire *et al.*, 1998) and the two researchers were awarded the 2006 Nobel Prize in Physiology or Medicine for their discovery. The work of Fire and Mello linked dsRNA to several

previously unexplained gene silencing phenomena and was the final piece in an RNA silencing puzzle that had been on-going for nearly a decade. The following description of events leading to the seminal paper of Fire and Mello is partially based on the review by Sen & Blau (2006).

The first report of an RNAi-like gene silencing phenomenon originated from studies of chalcone synthase (CHS), an enzymatic component of the pathway responsible for violet colouration in petunia flowers (petunias are cultivated hybrid plants, *Petunia* × *hybrida*, Family Solanaceae) (Napoli *et al.*, 1990). When researchers over-expressed CHS (by introducing a chimeric CHS gene) with the aim of generating violet petunia flowers, to their surprise they found that the resulting petunias were white. Analysis of transcript levels revealed that CHS messenger RNA (mRNA) levels in the transgenic petunias were fifty times lower than in the wild-type plants. The group hypothesised that this effect was caused by the exogenous transgene suppressing the endogenous CHS gene and they coined this process "co-suppression". The same phenomenon was observed by Romano & Macino (1992) when they transformed *Neurospora* (an ascomycete fungus) with portions of the *albino-3 (al-3)* and *albino-1 (al-1)* genes. Presence of the transgene caused low levels of expression of the endogenous *al-1* or *al-3* genes. The term "quelling" was used to describe this process in fungi.

Several years after these first reports of "co-suppression" and "quelling" in plants and fungi, an unexplained gene silencing phenomenon was observed in animals. Researchers working with the nematode worm, *Caenorhabditis elegans*, were attempting to knock down gene expression by introducing antisense RNA for the *PAR-1* (*partition* 1) gene (Guo & Kemphues, 1995). The use of antisense RNA, which was thought to act by hybridising with endogenous mRNA and either inhibiting translation or marking the RNA for degradation, was at the time thought to be potentially an attractive means of gene silencing. In addition to introducing antisense RNA in their experiments this group introduced sense RNA as a control. They were surprised to find that the sense RNA, which could not hybridise with endogenous mRNA, still targeted the *par-1* transcript for degradation.

In 1998 an explanation for these findings was forthcoming. Fire *et al.* (1998) hypothesised that the trigger for gene silencing was double-stranded RNA (dsRNA) and not single-stranded RNA (ssRNA). They explained the results of Guo & Kemphues (1995) in *C. elegans* by reasoning that the single-stranded sense and antisense RNA preparations used in their experiments may have been contaminated with dsRNA. Single-stranded RNA samples were generally prepared using bacteriophage RNA polymerases, which have been known to produce aberrant RNA molecules that may have double-stranded structure. This hypothesis

could also explain the “co-suppression” and “quelling” phenomena in plants and fungi, since transgene arrays can produce a fraction of aberrant (double-stranded) RNA products.

Fire *et al.* (1998) tested their hypothesis by studying interference towards the *C. elegans unc-22* gene, which encodes a myofilament protein. Decreases in the activity of this gene produce a twitching phenotype. During this study they compared the ability to interfere with endogenous gene expression of highly purified sense and antisense single-stranded RNA molecules with that of a mixture of sense and antisense RNA shown by gel electrophoresis to have hybridised in a double-stranded structure. Sense and antisense RNA preparations for *unc-22* had very little interference activity; whereas the double-stranded sense-antisense mixture produced effective interference with *unc-22* gene expression. They concluded that double-stranded RNA, and not single-stranded RNA, was the cause of gene silencing. They named this silencing phenomenon RNA interference (RNAi).

1.1.3 Interference by “dicing” and ”slicing”

The identification by Fire *et al.* (1998) of double-stranded RNA molecules as the gene silencing trigger led researchers to speculate as to the mechanism by which dsRNA directs the degradation of cognate endogenous mRNA. Hamilton & Baulcombe (1999) detected antisense RNAs 25 nucleotides in length, complementary to target mRNAs, in tissues during gene silencing experiments and hypothesised that these small RNAs could bind specifically to mRNA triggering its degradation or interfering with its translation. Further studies, conducted using a *Drosophila melanogaster* cell culture system, found that RNAi activity co-purifies with small RNA species (Hammond *et al.*, 2000) and that both strands of the introduced dsRNA are processed to RNA segments 21–23 nucleotides in length (Zamore *et al.*, 2000). The definitive link between silencing and small RNAs was made when researchers chemically synthesised dsRNAs 21–22 nucleotides in length and found that these molecules were able to provoke cleavage of the cognate mRNA of both heterologous and endogenous genes in *D. melanogaster* and mammalian cells (Elbashir *et al.*, 2001*a*, 2001*b*). These small RNA duplexes were termed short-interfering RNAs (siRNAs).

Following the identification of siRNAs as mediators of gene silencing the next step was to identify the enzymes responsible for the generation of siRNAs from dsRNA molecules (“dicing”) and the targeted cleavage of mRNA (“slicing”). Experiments in *Drosophila melanogaster* cells revealed that two separate enzymes were responsible for cleavage of dsRNA into siRNAs and cleavage of the target mRNA (Hammond *et al.*, 2000; Bernstein *et al.*, 2001). A candidate gene approach found that a type III RNase III enzyme possessed

dsRNA cleavage activity capable of yielding the characteristic 21–23 nucleotide siRNAs. This enzyme was named Dicer because of its dsRNA "dicing" activity. A co-immunoprecipitation study in mammalian cells identified two protein candidates for target mRNA cleavage (or Slicer) activity; Argonaute 1 and Argonaute 2 (Martinez *et al.*, 2002). It was unclear, however, whether the Argonaute enzymes were responsible for "slicer" activity (Martinez *et al.*, 2002) until Song *et al.* (2004) crystallized an Argonaute protein from the archaebacterium *Pyrococcus furiosus* and found remarkable similarity in the Argonaute PIWI (P-element induced wimpy testis) domain to the conserved secondary structure of RNase H enzymes, which act to hydrolyse the RNA of RNA/DNA hybrids (Cerritelli & Crouch, 2009). Moreover, "Slicer" activity, like RNase H activity, is dependent on divalent cations and leaves 3' OH and 5' phosphate termini (Martinez & Tuschl, 2004). Finally, immunoprecipitation of several tagged argonaute complexes revealed that only the Argonaute 2 (Ago-2) precipitate retained mRNA cleavage activity, indicating that it is this enzyme that has "Slicer" activity (Liu *et al.*, 2004).

1.1.4 Multiple RNAi pathways

In the decade following the identification of small RNAs as mediators of gene silencing, and of Dicer and Argonaute proteins as key components of the RNAi machinery, it has become clear that eukaryotes exhibit a number of gene silencing pathways involving small RNAs. In addition to the exogenous (or siRNA-mediated) RNAi pathway as described by Fire *et al.* (1998) and depicted in Figure 1.1, eukaryotic organisms are host to the endogenous RNAi pathway (mediated by micro RNAs; miRNAs), the piRNA (Piwi-interacting short RNA) pathway and RNAi mediated by endogenous siRNAs (esiRNAs) (Matranga & Zamore, 2007). Each pathway is thought to have evolved to play a distinct role; exogenous RNAi acts to defend against infecting viruses, endogenous RNAi functions as one of many controls on endogenous gene expression and piRNAs protect the genome against transposable elements (Obbard *et al.*, 2009).

In vitro studies with the model insect, *Drosophila melanogaster*, have revealed the mechanisms underlying the various RNAi pathways. Exogenous RNAi (Figure 1.2A), which is considered to have evolved as a defence against viral infection (Ding & Voinnet, 2007; Csorba *et al.*, 2009; Ding, 2010), takes place as follows. Exogenous dsRNAs (either experimentally introduced dsRNAs or viral RNAs) are processed by Dicer-2 into RNA duplexes 21-23 nucleotides in length with 2 nucleotide long 3' overhangs and 5' phosphate and 3' hydroxyl termini (named siRNAs, or viRNAs when they are derived from viruses; Ding & Voinnet, 2007) and loaded into the RNA-induced silencing complex (RISC) by

Dicer-2 with the involvement of an additional protein present in the complex, R2D2 (Liu *et al.*, 2003). One siRNA strand is released in an ATP-dependant manner and mRNA (or viral RNA) is cleaved by an Argonaute family protein following the binding of the remaining siRNA to its complementary sequence (Tolia & Joshua-Tor, 2007).

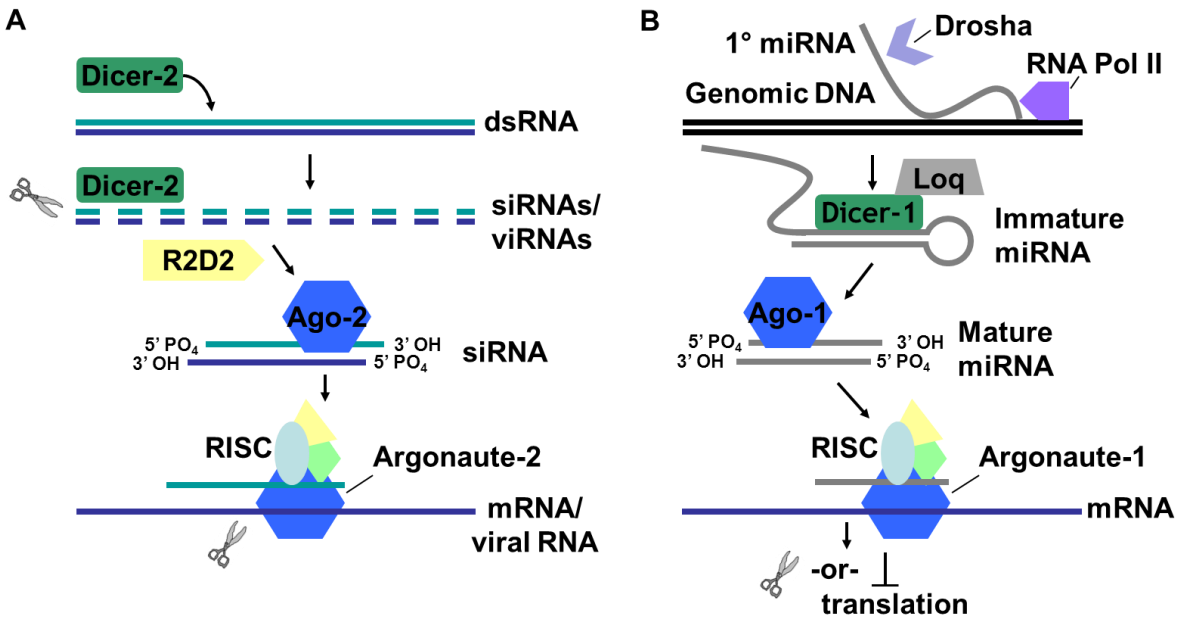


Figure 1.2: Multiple RNAi pathways. A Exogenous RNAi: exogenous dsRNAs are processed by Dicer-2 into short interfering RNAs (siRNAs), RNA duplexes 21-23 nucleotides in length with 2nt long 3' overhangs and 5' phosphate and 3' hydroxyl termini. siRNAs are loaded by Dicer-2 and R2D2 into an Argonaute-2(Ago-2)-containing complex (RISC; RNA-induced silencing complex). One strand of the siRNA is released and the remaining strand (the guide strand) binds to its complementary mRNA, which is cleaved by Argonaute 2. **B** Endogenous RNAi: Primary (1°) micro RNAs (miRNAs) are transcribed from the host genome by RNA Polymerase II (RNA Pol II). Following processing by Drosha, Dicer-1 and Loquacious (Loq) the mature miRNA becomes associated with an RNA-induced silencing complex (RISC), containing Argonaute 1 (Ago-1). One strand of the miRNA is released and the guide strand binds to its complementary messenger RNA (mRNA) leading to either cleavage of the mRNA or inhibition of its translation

Endogenous RNAi, which functions in the post-transcriptional regulation of eukaryotic gene expression (Carthew, 2006; Bushati & Cohen, 2007; Pillai *et al.*, 2007; Chapman & Carrington, 2007), proceeds via the following pathway (Figure 1.2B; described in reviews by Du & Zamore, 2005; Pillai *et al.*, 2007; Matranga & Zamore, 2007; Chapman & Carrington, 2007). In brief, micro RNAs (miRNAs) are encoded by host genomes; they are transcribed by RNA polymerase II into primary miRNAs and subsequently processed in the

nucleus by Drosha family proteins to form short stem loops (termed immature miRNAs). Immature RNAs are exported to the cytoplasm and are processed by another Dicer family protein, Dicer-1 and a dsRNA-binding domain (dsRBD) protein, Loquacious, to form the mature miRNA containing 5′ phosphate and 3′ hydroxy termini (Liu *et al.*, 2003). Mature siRNAs are loaded into the RNA-induced silencing complex (RISC), one strand of the miRNA duplex is released and the remaining strand binds to its complementary sequence in endogenous mRNA transcripts. Interestingly, from this point of the process onwards, a difference arises between plants and animals. In plants miRNAs match their mRNA targets exactly and binding results in cleavage and degradation of the target transcript (Obbard *et al.*, 2009). In animals miRNAs pair imperfectly with their target sequence and inhibit translation (Pillai *et al.*, 2007).

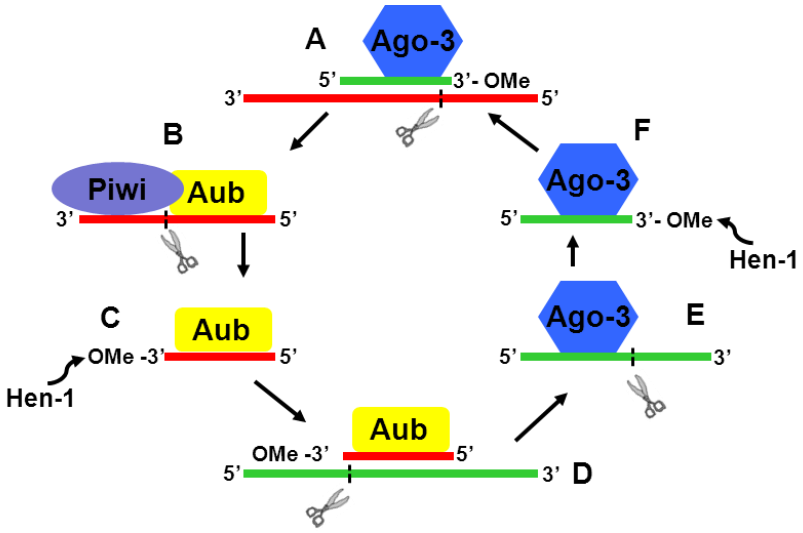


Figure 1.3: Model for the production of piRNAs in *Drosophila melanogaster*. A Sense piRNA (green) is bound by Argonaute 3 (Ago-3), which cleaves the target antisense transcript (red) to produce the 5′end of an antisense piRNA precurser. **B** The 3′ end of the piRNA precursor is trimmed by Aubergine (Aub) and Piwi. **C** Hen-1 methylates the 3′ end of the piRNA (3′OMe). **D** Aubergine-bound antisense piRNA cleaves the 5′ end of the sense transcript (green). The resulting sense piRNA precursor is bound by Ago-3 and **E** the 3′ end of the precursor is trimmed and **F** methylated by Hen-1. Diagram adapted from Klattenhoff & Theurkauf (2008).

The RNAi pathway mediated by Piwi-interacting short RNAs (piRNAs) targets transposable elements (TEs) and is described by Hartig *et al.* (2007), Aravin *et al.* (2007) and Klattenhoff & Theurkauf (2008). Unlike the exogenous and endogenous RNAi pathways, the piRNA pathway does not involve Dicer proteins, but it does rely on Argonaute proteins in the PIWI family. piRNAs, which are short RNAs with 5′ monophosphate and 2′-*O*-methyl, 3′ hydroxy termini, are thought to arise from loci rich in transposons and act to silence

dispersed copies of the selfish genetic elements present in the original trigger locus (Matranga & Zamore, 2007). Production of piRNAs is independent of Dicer proteins and it has been proposed that a cyclic feedback mechanism acts to generate piRNAs from their target transcripts (Figure 1.3; Brennecke *et al.*, 2007; Klattenhoff & Theurkauf, 2008).

Finally, the endogenous siRNA (esiRNA) pathway, which has only recently been discovered (Ghildiyal *et al.*, 2008; Chung *et al.*, 2008; Czech *et al.*, 2008; Kawamura *et al.*, 2008), appears to function to regulate host gene expression and to control TE transcript levels (Obbard *et al.*, 2009). The silencing target for esiRNAs is endogenously produced dsRNAs; either TEs, overlapping 3′-UTRs from genes on opposite strands or stem-loop (hairpin) structures. Targeting of endogenous dsRNAs occurs in a similar manner to the silencing of exogenous dsRNAs in the siRNA pathway (Figure 1.2) and involves Argonaute-2 and Dicer-2 (Chung *et al.*, 2008; Czech *et al.*, 2008).

In addition to the presence of multiple RNAi pathways mediated by different small RNA molecules, there is further variation in the mechanism or outcome of RNAi silencing. Interference can occur by post-transcriptional mechanisms including the cleavage and degradation of the target transcript (Obbard *et al.*, 2009) and the inhibition of translation (Pillai *et al.*, 2007) as well as by mechanisms acting prior to transcription including heterochromatin formation, DNA methylation, DNA elimination, or meiotic silencing by unpaired DNA (Pickford & Cogoni, 2003; Baulcombe, 2004; Matzke & Birchler, 2005; Meister & Tuschl, 2004; Ullu *et al.*, 2004; Zamore & Haley, 2005).

1.1.5 RNAi is conserved in eukaryotes

Since Fire's discovery in *C. elegans* in 1998, RNAi has been observed in a wide range of eukaryotic organisms. Eukaryotes can be divided into six supergroups; Excavata, grouping diplomonads and several genera of heterotrophic flagellates; Chromalveolata, including dinoflagellates, apicomplexan parasites, and the Stramenopiles; Archaeplastida, grouping red algae, green algae, and plants; Amoebozoa, including most traditional amoebae and slime moulds; Rhizaria, including the Foraminifera and the Cercozoa; and Opisthokonta, including animals and fungi (Medina, 2005; Adl *et al.*, 2005). By 2003, exogenous RNAi had been observed in five of the six groups for which data are available (Table 1.1; to my knowledge RNAi has not yet been confirmed in Rhizaria).

Table 1.1: Taxonomic distribution of exogenous RNAi in eukaryotes. Data are from Cerutti & Casas-Mollano (2006).

Supergroup	Species	Reference
Excavata	*Trypanosoma brucei*	Durand-Dubief & Bastin, 2003; Shi *et al.*, 2004
Chromalveolata	*Paramecium tetraurelia*	Galvani & Sperling, 2002
	Phytophthora infestans	Whisson *et al.*, 2005
Archaeplastida	*Chlamydomonas reinhardtii*	Rohr *et al.*, 2004; Schroda, 2006
	Arabidopsis thaliana	An *et al.*, 2003; Watson *et al.*, 2005
	Oryza sativa (japonica)	Miki & Shimamoto, 2004; Tang *et al.*, 2004
Amoebozoa	*Dictyostelium discoideum*	Martens *et al.*, 2002
	Entamoeba histolytica	Kaur & Lohia, 2004; Vayssié *et al.*, 2004
Rhizaria	-	Data not available.
Opisthokonta	*Schizosaccharomyces pombe*	Sigova *et al.*, 2004
	Neurospora crassa	Chicas *et al.*, 2005; Nakayashiki, 2005
	Aspergillus nidulans	Hammond & Keller, 2005
	Caenorhabditis elegans	Fire *et al.*, 1998; Grishok & Mello, 2002
	Anopheles gambiae	Keene *et al.*, 2004; Hao *et al.*, 2008
	Drosophila melanogaster	Kennerdell & Carthew, 1998; Piccin *et al.*, 2001
	Danio rerio	Wargelius *et al.*, 1999
	Xenopus laevis	Oelgeschlager *et al.*, 2000
	Homo sapiens	Elbashir *et al.*, 2001*a*; Paddison *et al.*, 2002

The presence of functioning exogenous RNAi pathways in such a wide range of eukaryotic organisms suggests that the last common ancestor of the eukaryotes was capable of RNAi. Further evidence to support this theory is provided by the taxonomic distribution of RNAi machinery components. Cerutti & Casas-Mollano (2006) surveyed genomes belonging to five of the six eukaryotic supergroups and found Argonaute-Piwi and Dicer-like proteins in

members all of five groups. It is likely, therefore, that relatively complex RNAi machinery was already present in the last common ancestor of the eukaryotes. They concluded that this ancestral RNAi machinery likely consisted of, at least, one Argonaute-like polypeptide, one Piwi-like protein, one Dicer and one RNA-dependent RNA polymerase.

Despite the widespread presence of RNAi proteins, they do appear to have been lost from specific lineages and seem to be entirely absent in *Trypanosoma cruzi* and *Leishmania major*, organisms which appear to be deficient in RNAi (Robinson & Beverley, 2003; DaRocha *et al.*, 2004; Ullu *et al.*, 2004). Furthermore, there are differences in susceptibility to RNAi amongst those organisms which are capable of exogenous RNAi. One key difference in the competency of organisms to carry out RNAi is their ability to amplify the siRNA pool using a host-encoded RNA-dependent RNA polymerase (RdRp) and propagate the signal to other cells (Jose & Hunter, 2007; Chapman & Carrington, 2007). Amplification and spread of the RNAi signal allows silencing to occur in tissues distal to the site of dsRNA delivery, a phenomenon known as systemic or environmental RNAi (Whangbo & Hunter, 2008).

Nematode worms and plants both encode RdRps, which act to propagate the signal via different mechanisms. In *Caenorhabditis elegans*, RdRp binds the siRNA-target duplex and directly synthesises secondary siRNAs (Sijen *et al.*, 2007; Pak & Fire, 2007), whereas in plants, RdRp synthesises long dsRNAs, which are then processed into siRNAs by a Dicer family protein (Himber *et al.*, 2003; Moissiard *et al.*, 2007). RdRPs, however, are not conserved in all eukaryotic lineages and are not present in the genomes of insects or vertebrates (Gordon & Waterhouse, 2007; Obbard *et al.*, 2009).

1.1.6 Applications of RNAi

RNAi has proved itself to be a powerful tool for investigating gene function in eukaryotes (Dykxhoorn & Lieberman, 2005), since the experimental introduction of RNAi triggers can interfere with gene expression in a targeted manner. Although silencing is typically incomplete and the term “knockdown” and not “knockout” is used to describe the interference with gene expression (Dykxhoorn & Lieberman, 2005), silencing by RNAi is sufficiently effective to be useful in reverse genetics studies. Indeed, in some cases, RNAi can be extremely effective with the targeted mRNA becoming undetectable following RNAi even with ultrasensitive PCR assays (Song *et al.*, 2003*a*). Furthermore, the silencing effect is often long-lasting; siRNA-mediated silencing can persist for several weeks in terminally differentiated, nondividing cells, such as macrophages or neurons (Song *et al.*, 2003*b*; Omi

et al., 2004) and interference is frequently sufficiently long-lasting to allow (for example) developmental perturbation when key genes are silenced.

RNAi has proved useful for reverse genetics studies in many eukaryotic taxa and has allowed advances in many fields of biology (see Table 1.1 for studies which have utilised RNAi to study gene function). There are, however, some key differences in the experimental methods employed to silence genes in different taxa, in particular with regards to the RNAi trigger. In *C. elegans* and many other invertebrate groups, including insects, long double-stranded RNA (dsRNA) molecules are capable of inducing gene silencing (Fire *et al.*, 1998; Kennerdell & Carthew, 1998) and are typically used as the RNAi "trigger" during RNAi experiments. In contrast, long dsRNA is destroyed in mammals by the antiviral interferon response (Stark *et al.*, 1998) and it is therefore necessary to introduce synthetic siRNAs to induce a knockdown (Elbashir *et al.*, 2001*a*, 2001*b*). Consequently, siRNAs, as well as DNA vectors which express siRNAs or siRNA precursors (for example, shRNAs; small-hairpin RNAs), are the stimulus of choice in mammalian RNAi experiments (Dorsett & Tuschl, 2004).

In addition to investigating the function of individual genes in reverse genetics studies, RNAi has also been used to silence gene expression on a whole-genome scale to functionally annotate genomes (Shan, 2010). In *C. elegans* a whole genome RNAi feeding library (Kamath *et al.*, 2001; Simmer *et al.*, 2003) has been used to identify genes in crucial biological pathways such as fat regulation, aging and miRNA function (Ashrafi *et al.*, 2003; Samuelson *et al.*, 2007; Parry *et al.*, 2007). RNAi screening in *C. elegans* is facilitated by the susceptibility of the organism to RNAi: knockdowns can be achieved by simply soaking the animals in dsRNA or feeding them bacteria that produce dsRNA (Tabara *et al.*, 1998; Timmons & Fire, 1998). In *Drosophila melanogaster* RNAi is ineffective in most tissues, probably caused by a deficiency in dsRNA uptake (Miller *et al.*, 2008) and therefore the majority of RNAi screens are performed with S2 cells, which are able to readily internalise dsRNAs (Ulvila *et al.*, 2006). RNAi screening in S2 cells has identified genes involved in cytokinesis, cell cycle regulation, specific signalling pathways and virus replication (Bjorklund *et al.*, 2006; DasGupta & Gonsalves, 2008; Mattila *et al.*, 2008; Bonaldi *et al.*, 2008; Hao *et al.*, 2008). Whole organism RNAi screening in *D. melanogaster* has also become possible due to the availability of a genome-wide library of RNAi transgenes for conditional gene inactivation (Dietzl *et al.*, 2007). RNAi transgenes contain an inverted repeat sequence, which, following transcription, is processed into a long dsRNA that is finally diced into siRNAs. The RNAi transgene can be conditionally expressed, or expression targeted to chosen tissues, by putting the transgene under the control of an

appropriate promoter. This allows the screening of genes playing roles in neurons and other cell types at the whole animal level (Cauchi *et al.*, 2008; Liu & Davis, 2009).

Screening of mammalian genes using RNAi typically takes place in cell culture, with siRNA libraries, plasmid-based shRNA libraries, and virus-based shRNA libraries being available for screening mouse or human cells (Chang *et al.*, 2006; Root *et al.*, 2006; Fewell & Schmitt, 2006; Ganesan *et al.*, 2008). Genome-wide RNAi-based screens have identified novel genes involved in pigmentation, cell division, cell signalling and virus replication (Kittler *et al.*, 2007; Zhou *et al.*, 2008*a*; Ganesan *et al.*, 2008). RNAi screens have also proved useful in the discovery of novel drug targets, since they enable the identification of proteins crucial to disease pathogenesis (Iorns *et al.*, 2007). For instance, Collins *et al.* (2006) screened a library of siRNAs for their ability to block the migration of a highly motile ovarian carcinoma cell line and found that siRNAs against MAP4K4 (mitogen-activated protein kinase kinase kinase kinase 4) inhibited migration of the cells. Further studies showed that the knockdown of this transcript inhibited the migration of multiple carcinoma cell lines. Tissue invasion and metastasis are important phenotypic characteristics of tumour cells (Hanahan & Weinberg, 2000): gene products essential for cell migration, such as MAP4K4, may, therefore, represent potentially relevant therapeutic targets.

In addition to its impact in experimental biology and drug discovery there has been considerable interest in developing RNAi as a therapeutic tool (Vaishnaw *et al.*, 2010). Following the discovery that siRNAs could induce RNAi in mammalian cells (Elbashir *et al.*, 2001*b*) small RNA-mediated silencing of disease-associated targets was recognised as a potentially useful therapeutic approach. From a pharmacological perspective small RNAs were considered to be particularly attractive therapeutic molecules due to the specificity and potency of their silencing activity (Hannon & Rossi, 2004; Karagiannis & El-Osta, 2005). Following the recognition of small RNAs as potentially useful pharmacological molecules there has been a large research effort directed at developing potential RNAi therapeutics for conditions such as viral infections, cancer and neurodegenerative diseases (Karagiannis & El-Osta, 2005). In particular, efforts have been focused on diseases with a high degree of unmet medical need (Vaishnaw *et al.*, 2010).

Despite evidence of the considerable potential of RNAi-mediated therapies there have been several major obstacles to their development, including inefficient *in vivo* delivery and uncertainty about potential toxicity (Dorsett & Tuschl, 2004; Hannon & Rossi, 2004). However, both local and systemic delivery has been demonstrated pre-clinically (de Fougerolles *et al.*, 2007; Novobrantseva *et al.*, 2008; Whitehead *et al.*, 2009) and numerous

on-going clinical trials have so far given no indication of serious adverse effects linked to siRNA exposure (Vaishnaw *et al.*, 2010). At the time of preparation of this thesis (September 2011) there are twelve RNAi therapeutic products in clinical trials, mostly in phase I or phase II. The most advanced RNAi therapeutic program in clinical practise, currently in phase 2b, involves targeting of the respiratory syncytial virus (RSV) N transcript with an unmodified siRNA. In a double-blind, randomised, placebo-controlled study prophylactic intranasal treatment with siRNA was shown to decrease the incidence of experimental upper respiratory tract infection with RSV (Vaishnaw *et al.*, 2010).

1.2 RNAi in insects

1.2.1 Initial RNAi experiments in insects

Shortly following the discovery of RNAi in nematode worms, successful RNAi experiments were conducted in insects. In 1998, Kennerdell and colleagues reported potent and specific interference of gene expression following injection of double-stranded RNA (dsRNA) into *Drosophila melanogaster* syncytial blastoderm embryos (Kennerdell & Carthew, 1998). In an initial proof-of-concept experiment they targeted the *fushi tarazu* (*ftz*) and *even-skipped* (*eve*) genes, which are required for embryonic segmentation, and found that injection of dsRNA into wild-type embryos effectively interfered with gene activity as demonstrated by cuticle phenotypes characteristic of *ftz* or *eve* mutants. Following successful interference with *wingless*, which is required for the determination of specific cell fates and polarity within each segmental unit of the embryo, the researchers used RNAi to interfere with expression of the *frizzled* and *frizzled 2* genes, demonstrating that they belong to the *wingless* pathway.

In 1999 Brown and colleagues confirmed that RNAi was effective in *Drosophila melanogaster* and showed that *Tribolium castaneum* was also sensitive to RNAi (Brown *et al.*, 1999). In this study dsRNA targeted to the homeotic gene *Deformed* (*Dfd*) was injected into *Drosophila melanogaster* and *Tribolium castaneum* embryos and was successful in producing the well characterised *Deformed* mutant phenotype in both insects. Further successful RNAi studies were published in the following year, including that of Stauber *et al.* (2000), which used RNAi to study the function of Bicoid and Hunchback in embryos of the lower cyclorrhaphan fly, *Megaselia abdita,* and a study which used RNAi to target the Homeobox (Hox) genes *Deformed* (*Dfd*), *Proboscipedia* (*pb*) and *Sex combs reduced* (*Scr*) in the milkweed bug, *Oncopeltus fasciatus* (Hughes & Kaufman, 2000).

1.2.2 Cellular mechanism of insect RNAi

Because *in vitro* studies in *Drosophila melanogaster* have been of key importance in determining the steps involved in RNAi, the description of the "general" exogenous RNAi pathway in section 1.1.4 and Figure 1.2A, is valid when we are discussing insect RNAi. In recent years a more complete picture of silencing by exogenous RNAi has developed. In particular, our understanding of the assembly of the RNA-induced silencing complex (RISC), which functions to guide siRNAs to their target RNA, has improved. In *D. melanogaster*, it is proposed that siRNAs are loaded on to RISC via an RLC (RISC-loading complex), which contains Dicer-2 (Dcr-2) and a partner protein R2D2 (Figure 1.4; Tomari & Zamore, 2005). Liu *et al.* (2009) found that the addition of recombinant Dcr-2 and R2D2 to recombinant Ago2 and duplex siRNA induced RISC activity (and mRNA cleavage). They subsequently identified a RISC enhancer named C3PO (component 3 promoter of RISC), consisting of translin and TRAX (translin-associated factor-X), that was required in *D. melanogaster* for *in vivo* RNAi. The group found that C3PO possessed ribonuclease activity and proposed that that C3PO associates with Dcr2–R2D2 during RLC activity to form the active RISC complex and serves within this complex to remove the passenger strand of the duplex siRNA, freeing the guide strand to target Ago2 to the target mRNA.

1.2.3 RNAi as a tool for analysis of gene function in insects

The discovery that RNAi was effective in insects was welcomed by the insect research community. Prior to the advent of RNAi technologies it was difficult to perform any analysis of gene function outside of the few tractable genetic model insects such as *Drosophila melanogaster* and *Tribolium castaneum* (Hughes & Kaufman, 2000). Furthermore, genome data has become more readily available (Bellés, 2010) due to advances in DNA sequencing technologies that have reduced the cost of sequencing by over two orders of magnitude (Shendure & Ji, 2008). This growing availability of genomes has revealed a large array of genes with unknown functions, leading to the problem of how to unveil the functions of these new genes (Bellés, 2010). Insect RNAi has allowed the analysis of gene function in non-model insects (Mito *et al.*, 2011) and has been readily utilised to investigate gene function in insects.

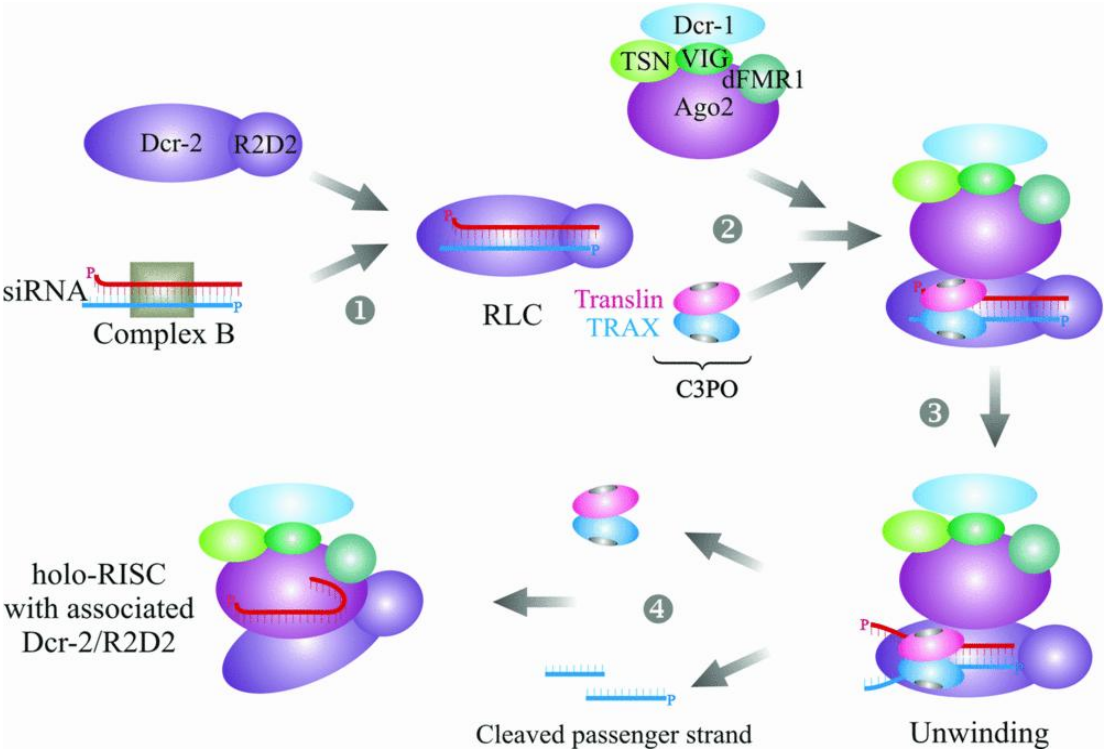


Figure 1.4: The RISC assembly pathway for exogenous RNAi in *Drosophila melanogaster*. Step 1: the siRNA duplex is transferred from complex B to the RLC, consisting of Dcr-2 and R2D2. Step 2: C3PO (translin and TRAX) are joined with the RLC and the RISC complex {consisting of the Dcr-1, TSN (Tudor-staphylococcal nuclease), VIG (vasa intronic gene), dFMR (*Drosophila* FMR) and Ago2 subunits} to generate the holoRISC by a Drc-2–Ago2 interaction. Step 3: the passenger strand is removed/endonucleolytically cleaved from the siRNA, which is enhanced by C3PO activity. Step 4: the holoRISC complex can proceed to associate with target mRNAs. It remains unclear whether C3PO remains associated with the holoRISC, or whether it dissociates, as depicted in the model shown. The schematic and text in this figure are reproduced from Jaendling & McFarlane (2010).

It would be extremely difficult to document all of the studies which have employed RNAi to investigate gene function in insects because of the large number of publications in the field. A Web of Science search with "RNA interference* and insect*" as the search query returns 380 articles from 2010 alone. Not only have there been many publications reporting the use of RNAi, but these papers also concern a broad range of insect species. In just one year from July 2008-June 2009 effective RNAi experiments were reported from the dipterans *Aedes aegypti* (Xi *et al.*, 2008), *Anopheles gambiae* (Magalhaes *et al.*, 2008), *Armigeres subalbatus* (Tsao *et al.*, 2009), *Culex pipiens* (Sim & Denlinger, 2009), *Drosophila melanogaster* (Miller *et al.*, 2008), *Episyrphus balteatus* (Lemke & Schmidt-Ott, 2009) and *Glossina morsitans morsitans* (Walshe *et al.*, 2009), the lepidopterans *Bombyx mori* (Hossain *et al.*, 2008), *Manduca sexta* (Eleftherianos *et al.*, 2009*b*), *Plutella xylostella* (Bautista *et al.*, 2009)

and *Spodoptera litura* (Chen *et al.*, 2008), the coleopterans *Onthophagus taurus* and *Onthophagus binodis* (Moczek & Rose, 2009) and *Tribolium castaneum* (Angelini *et al.*, 2009), the hymenopterans *Apis mellifera* (Marco Antonio *et al.*, 2008) and *Solenopsis invicta* (Lu *et al.*, 2009), the blattarian *Blattella germanica* (Lee *et al.*, 2009) and the orthopteran *Gryllus bimaculatus* (Moriyama *et al.*, 2008). In support of these observations Bellés (2010) conducted a comprehensive review of the insect RNAi literature and reported successful RNAi in many endopterygote (Coleoptera, Neuroptera, Hymenoptera, Lepidoptera and Diptera) and exopterygote (Hemiptera, Orthoptera, Blattaria and Isoptera) Orders.

Not only has effective RNAi has been demonstrated in many insect species, but is has also been performed in insects in a variety of developmental stages. The initial insect RNAi experiments involved the induction of gene silencing in embryos and many subsequent studies have investigated gene function in this developmental stage (Kennerdell & Carthew, 1998; Stauber *et al.*, 2000; Lorenzen *et al.*, 2002; Herke *et al.*, 2005; Mito *et al.*, 2005; Angelini *et al.*, 2005; Kuwayama *et al.*, 2006; Hasselmann *et al.*, 2008; Liu *et al.*, 2008; Lemke & Schmidt-Ott, 2009; Pan *et al.*, 2009; Grossmann *et al.*, 2009). RNAi experiments have not been restricted to embryos, however. Following experiments in *Tribolium castaneum* and *Spodoptera litura* which demonstrated that RNAi could be used to create loss-of-function phenotypes in larvae, pupae and adult insects (Rajagopal *et al.*, 2002; Tomoyasu & Denell, 2004), there have been many examples of larval, nymphal and adult RNAi (Dong & Friedrich, 2005, 2010; Tomoyasu *et al.*, 2005; Arakane *et al.*, 2005; Martín *et al.*, 2006; Araujo *et al.*, 2006; Nakamura *et al.*, 2007; Gandhe *et al.*, 2007*a*, 2007*b*; Soberón *et al.*, 2007; Minakuchi *et al.*, 2008; Niu *et al.*, 2008; Kim *et al.*, 2008; Xi *et al.*, 2008; Magalhaes *et al.*, 2008; Moriyama *et al.*, 2008; Marco Antonio *et al.*, 2008; Ohnishi *et al.*, 2009; Tsao *et al.*, 2009; Moczek & Rose, 2009; Sim & Denlinger, 2009; Lu *et al.*, 2009; Hamada *et al.*, 2009)

RNAi experiments in insects typically involve the introduction of the RNAi trigger into a particular developmental stage of the insect and the confirmation of a reduction in gene expression and observations of the resulting phenotype in that same developmental stage. However, researchers have occasionally introduced the RNAi trigger into a preceding developmental stage. Tomoyasu & Denell (2004) demonstrated that RNAi could be used to create pupal and adult loss-of-function phenotypes in the red flour beetle, *Tribolium castaneum*, by injection of double-stranded RNA (dsRNA) into late instar larvae. They found that injected green fluorescent protein (GFP) dsRNA can inhibit GFP expression beginning shortly after injection and continuing through pupal and adult stages and that

larval RNAi of the *Tc-achaete-scute-homolog* gene, which encodes a protein that functions in the development of adult sensory bristles, could induce morphological defects in adult beetles. Parental RNAi, where dsRNA is introduced into adult insects in order to interfere with gene expression in subsequent embryos, has also been effectively employed (Bucher *et al.*, 2002; Bettencourt *et al.*, 2002; Liu & Kaufman, 2004; Mito *et al.*, 2005; Lynch & Desplan, 2006; Khila & Grbić, 2007; Ronco *et al.*, 2008)

In RNAi studies of gene function in insects the trigger is typically introduced by injection (for example in larvae and adults Rajagopal *et al.*, 2002; Arakane *et al.*, 2005; Tsao *et al.*, 2009 and in embryos Liu *et al.*, 2008; Lemke & Schmidt-Ott, 2009; Pan *et al.*, 2009). Injections, however, are not always easy to administer and may induce mortality in the test insects (in particular if the injection is made into early stage larvae or embryos; Bucher *et al.*, 2002). There has, therefore, been interest in achieving RNAi by oral delivery of the RNAi trigger, since this is a less invasive technique. Successful oral RNAi was first demonstrated by Turner *et al.* (2006), who fed larvae of the light brown apple moth, *Epiphyas postvittana*, dsRNA for a larval gut carboxylesterase gene and found a substantial reduction in carboxylesterase transcript levels after two days. Araujo *et al.* (2006) subsequently found that feeding the triatomine bug, *Rhodnius prolixus*, dsRNA for *nitrophorin 2* silenced gene expression. Further successful oral RNAi experiments include those conducted by Meyering-Vos & Müller (2007), Zhao *et al.* (2008), Zhou *et al.* (2008*b*), Bautista *et al.* (2009), Shakesby *et al.* (2009), Tian *et al.* (2009), Walshe *et al.* (2009), Whyard *et al.* (2009), Upadhyay *et al.* (2011) and Zhu *et al*. (2011).

Insect RNAi has been used as a tool to analyse gene function in a number of biological fields. The review by Bellés (2010) contains a table consisting of functions studied by systemic RNAi in insects and therefore I do not make a comprehensive study here. However, a list of the studied functions in lepidopteran species is presented in Table 1.2, giving an indication of the wide array of fields for which RNAi has been employed.

1.2.4 RNAi for insect pest control

Another potential application of insect RNAi is in the field of pest control. Insect pests represent a major problem; they not only cause significant crops losses (pre-harvest losses of 18% worldwide; Oerke, 2006), but also act as vectors of important human diseases such as malaria, dengue, trypanosomiasis, filariasis, leishmaniasis, and Chagas disease (Lambrechts *et al.*, 2009). Due to the problems associated with the use of the "traditional" chemical insecticides (including effects on human health, agroecosystems, nontarget

species,landscapes and communities and the selection of insecticide-resistant traits in pest species; Devine & Furlong, 2007) there is a need to develop novel insect pesticides.

Table 1.2: Functions studied using RNAi in lepidopteran insects. Adapted from Terenius *et al.* (2011).

Studied function	**References**
Bacillus thuringiensis toxin receptors	Rajagopal *et al.*, 2002; Sivakumar *et al.*, 2007; Soberón *et al.*, 2007; Yang *et al.*, 2010
Chitin synthesis pathway	Chen *et al.*, 2008; Tian *et al.*, 2009
Circadian rhythm of sperm release	Gvakharia *et al.*, 2003; Kotwica *et al.*, 2009
Embryonic development	Bettencourt *et al.*, 2002; Fabrick *et al.*, 2004; Tsuzuki *et al.*, 2005; Mrinal & Nagaraju, 2008; Liu *et al.*, 2008; Tomita & Kikuchi, 2009; Masumoto *et al.*, 2009
Insecticide resistance	Bautista *et al.*, 2009
Immune system	Hirai *et al.*, 2004; Levin *et al.*, 2005; Eleftherianos *et al.*, 2006*b*, 2006*c*, 2006*a*, 2007*a*, 2007*b*, 2009*b*, 2009*a*; Terenius *et al.*, 2007; Zhuang *et al.*, 2007*a*, 2007*b*, 2008; Gandhe *et al.*, 2007*a*, 2007*b*
Pesticide development	Whyard *et al.*, 2009
Pheromone binding	Turner *et al.*, 2006
Postembryonic development	Gui *et al.*, 2006; Meyering-Vos *et al.*, 2006; Huang *et al.*, 2007; Griebler *et al.*, 2008; Dai *et al.*, 2008; Hossain *et al.*, 2008; Kumar *et al.*, 2009*a*; Yang *et al.*, 2009; Tang *et al.*, 2010*a*; Khajuria *et al.*, 2010; Shukla & Nagaraju, 2010
Sex pheromone synthesis	Ohnishi *et al.*, 2006, 2009; Hull *et al.*, 2009, 2010
Trehalose synthesis pathway	Chen *et al.*, 2010*a*, 2010*b*; Tang *et al.*, 2010*b*

RNAi-based gene silencing could represent a novel insecticide technology, since it is theoretically possible to protect plants against insect herbivores by downregulating the expression of essential genes in the pest (Price & Gatehouse, 2008). The observation that RNAi can be triggered in some insect species by diet containing dsRNA (Meyering-Vos & Müller, 2007; Zhou *et al.*, 2008*b*; Tian *et al.*, 2009; Bautista *et al.*, 2009; Shakesby *et al.*, 2009; Walshe *et al.*, 2009; Whyard *et al.*, 2009; Zhu *et al.*, 2011; Upadhyay *et al.*, 2011) made this technology feasible, since oral administration represents a likely delivery route for an RNAi pesticide (Gordon & Waterhouse, 2007). Combined with the demonstration of transgene-encoded RNAi in plants (Waterhouse *et al.*, 1998), this prompted speculation that plants could be protected from herbivorous insects by engineering them to express dsRNAs targeting vital insect genes (Gordon & Waterhouse, 2007).

The potential of this technology was revealed when two seminal papers were published in 2007 (reviewed by Gordon & Waterhouse, 2007). Baum *et al.* (2007) demonstrated that transgenic corn plants engineered to express western corn rootworm, *Diabrotica virgifera virgifera*, dsRNAs specific to genes with essential functions show a reduction in insect feeding damage. Another group achieved *in planta* RNAi using cotton bollworm (*Helicoverpa armigera*); they engineered plants to express siRNAs specific to a gene required for the insect to tolerate the cotton metabolite gossypol. They achieved reductions in transcript levels of the target gene and retardation of insect growth rate (Mao *et al.*, 2007). These results show that transgene-encoded ingestible dsRNA may in principle be utilised in the control of insect pests, although of course this is not to say that such RNAi-based pesticides will be cost-effective.

Since 2007 many potential targets for RNAi insecticides have been identified. Ohde *et al.* (2009) found that by using RNAi to interfere with *vestigial* and *scalloped*, genes with roles in wing formation, in larvae of the 28-spotted potato ladybird, *Henosepilachna vigintioctopunctata*, they were able to produce flightless beetles. Tang *et al.* (2010*a*) targeted two storage hexamerins, important members of the hemocyanin superfamily, which likely function as sources of amino acids during metamorphosis, reproduction and development, in *Spodoptera exigua*. Injection of *S. exigua hexamerin 1* and *storage protein 1* dsRNA caused significantly more mortality than the control injections. Walker III & Allen (2011) injected dsRNA for the *inhibitor of apoptosis* gene into nymphs and adults of the tarnished plant bug, *Lygus lineolaris*, and observed significantly reduced lifespan compared with those injected with non-insect dsRNA (for eGFP). Zhao *et al.* (2011) identified a potential RNAi target in the striped flea beetle, *Phyllotreta striolata*. They injected dsRNA for an odorant receptor

(*PsOr1*) into final-phase pupae and the subsequent adult beetles were unable to sense the attractant or repellent odour stimulus and showed impaired host-plant preference.

The above described studies interfered with the expression of potential insecticide targets following the introduction of RNA triggers by injection. The following studies not only identified potential targets for interference but also demonstrated that they were able to interfere with expression by oral RNAi. Zhao *et al.* (2008) fed dsRNA targeting *arginine kinase*, a phosphotransferase with a critical role in cellular energy metabolism, to *Phyllotreta striolata*, a destructive beetle pest, and observed increased mortality in the beetles. Tian *et al.* (2009) demonstrated that silencing of genes not expressed in the midgut following ingestion of insect dsRNA was possible. They showed that the development of *Spodoptera exigua* larvae fed *Escherichia coli* expressing dsRNA of *chitin synthase gene A*, which is expressed in the cuticle and tracheae, was disturbed, resulting in lethality. Whyard *et al.* (2009) fed species-specific dsRNA targeting *vATPase* transcripts to fruit flies (*Drosophila melanogaster*), flour beetles (*Tribolium castaneum*), pea aphids (*Acyrthosiphon pisum*), and tobacco hornworms (*Manduca sexta*) and induced mortality in the insects. Chen *et al.* (2010*b*) targeted the gene *trehalose phosphate synthase* in the brown planthopper, *Nilaparvata lugens*, a rice pest. They found that the development of *N. lugens* larvae that had been fed with the dsRNA was disturbed, resulting in lethality. Zhu *et al.* (2011) fed dsRNA for five target genes (*β-Actin, Protein transport protein sec23, Vacuolar ATP synthase subunit E, Vacuolar ATP synthase subunit B* & *Coatomer subunit beta*) to the Colorado potato beetle, *Leptinotarsa decemlineata*, a pest of potatoes, tomato and eggplant, and observed significant mortality and reduced weight gain in the treated beetles. Finally, Upadhyay *et al.* (2011) targeted five different genes (*actin ortholog, ADP/ATP translocase, alpha-tubulin, ribosomal protein L9* & *V-ATPase A* subunit) in the whitefly, *Bemisia tabaci*, and reported mortality in the insect after six days of feeding.

An interesting alternative to oral delivery has recently been considered. Pridgeon *et al.* (2008) attempted to silence *Aedes aegypti* genes using dsRNAs delivered topically in an effort to develop new insecticides to control mosquitoes. They found that topical application of dsRNA for the *inhibitor of apoptosis protein 1* gene (*AaeIAP1*) was able to kill adult female mosquitos. Subsequently, Wang *et al.* (2011) sprayed dsRNAs for *chymotrypsin-like serine protease C3* (DS10) and an unknown protein (DS28) onto newly hatched *Ostrinia furnalalis* larvae resulting in around 40-50% mortality. Investigations with topically applied fluorescent dsRNAs confirmed that dsRNA did penetrate the body wall and circulate in the body cavity (Wang *et al.*, 2011).

1.2.5 Susceptibility to RNAi

RNAi has, clearly, been extensively utilised in insects. However, not all species show the same degree of sensitivity toward RNAi (Bellés, 2010). It appears that insensitivity to RNAi may be taxon-specific. For instance, Terenius *et al.* (2011) state that performing RNAi in Lepidoptera is not as straight-forward as for other insects and took steps to investigate this phenomenon by integrating published and unpublished results. Bellés (2010) suggests some potential causes of RNAi insensitivity, including reasons intrinsic to the species, the tissue or the gene. Possible reasons relating to the species include that the alien dsRNA is efficiently degraded, that there is deficient amplification and spreading of the RNA signal, and that there is a low response of core RNAi genes after dsRNA treatment (Bellés, 2010).

In this study we have experienced difficulties achieving RNAi in the tobacco hornworm, *Manduca sexta* (Lepidoptera). This thesis explores several possible causes for the insensitivity of insects to RNAi using *Manduca sexta* as a case study. Figure 1.5 highlights three key phases of a successful RNAi experiment, which, as identified by Bellés (2010), may be deficient in RNAi-insensitive insect species. These three phases were investigated during this thesis for their likely contribution to the observed insensitivity of *M. sexta* to RNAi.

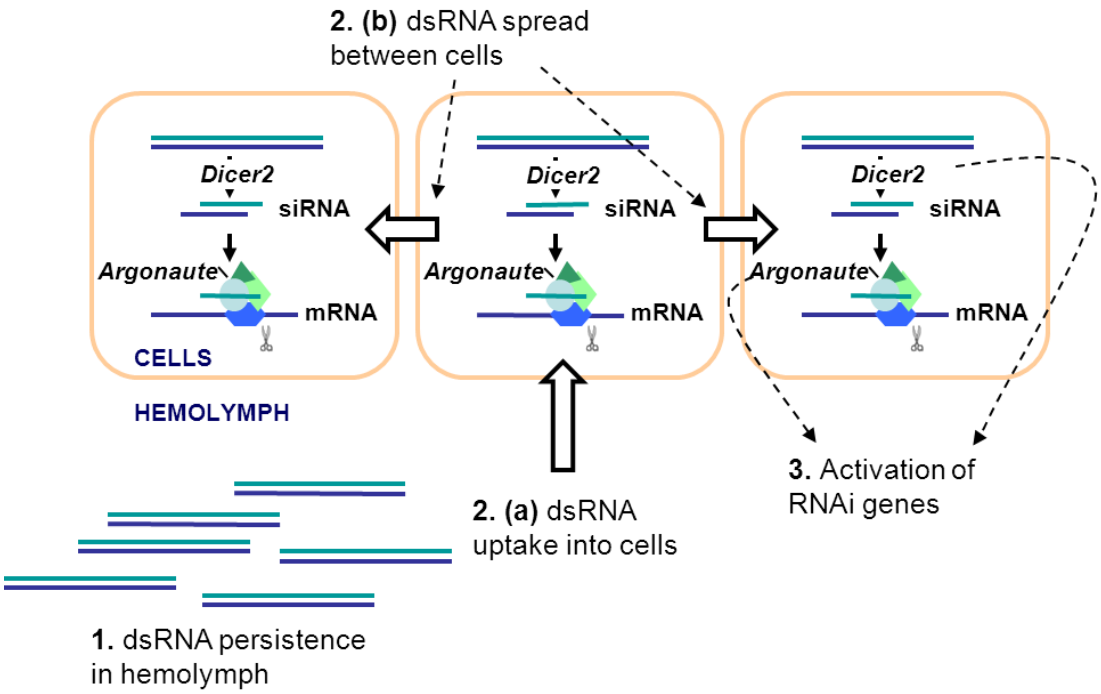


Figure 1.5: Phases in a successful RNAi experiment (where dsRNA is introduced by hemocoel injection).

In order to have a point of comparison experiments were also conducted in the German cockroach, *Blattella germanica* (Blattodea), which, unlike *M. sexta*, was found to be sensitive to RNAi. In Chapter Three of this dissertation I present examples of RNAi experiments in *M. sexta* and *B. germanica* in order to highlight the disparity in their sensitivity to RNAi. In Chapter Four I investigate the survival of dsRNA in the hemolymph of *B. germanica* and *M. sexta* and report that synthetic dsRNA molecules are rapidly degraded in *Manduca sexta* hemolymph, while there appears to be no degradation of dsRNA following incubation in the hemolymph of *B. germanica*. In Chapter Five I explore whether inadequate uptake into cells is responsible for the insensitivity of *Manduca sexta* to RNAi and find no evidence for a lack of uptake of dsRNA into *Manduca sexta* tissue. Chapter Six considers whether inadequate expression of several core RNAi genes (including *Dicer-2* and *Argonaute-2*) is linked to the insensitivity of *M. sexta* to RNAi and reports that not only are *Dicer-2* and *Argonaute-2* expressed at relatively high levels in *M. sexta* tissue, but that transcript levels are elevated in response to injection with dsRNA.

CHAPTER 2: GENERAL METHODS

2.1 Insect culture

Larvae of the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), were reared at the University of Bath according to the instructions of Bell & Joachim (1976) and Reynolds *et al* (1985). Caterpillars were kept at 25°C, 50% humidity and a photoperiod of 17h light: 7h dark. Artificial diet was prepared according to the recipe of Yamamoto (1969) as modified by Bell & Joachim (1976). Newly hatched larvae were transferred individually to the surface of a piece of diet in a plastic container (approximately 30 ml in volume) with a tight-fitting lid. The diet was not changed during the first four instars (12-13 days). On the day of ecdysis to the fifth (and final) instar the newly emerged fifth instar larvae were transferred to fresh diet in a larger container (volume approximately 200 ml). Wandering fifth instar larvae were transferred into wooden blocks for 10 days to pupate, after which they were removed. Approximately 20 days after pupation pupae on the verge of emergence were transferred to the adult cage, where the adult moths emerged and climbed a wire mesh to inflate and stiffen their wings. Moths were fed on a diet of a 10% sucrose solution supplied in a plastic "artificial flower" and laid eggs on an "artificial leaf" composed of a flat wooden plate covered with a nappy liner. Nappy liners were removed to the insectarium daily and eggs hatched into larvae after four days. Newly moulted (day 0) fifth instar larvae were used for all experiments unless otherwise stated.

Blattella germanica adults were obtained from a colony kept at the CSIC-UPF Instituto de Biología Evolutiva, Barcelona, Spain, which was fed on Panlab dog chow and water, and reared in complete darkness at 30±1°C and 60–70% relative humidity.

2.2 Insect manipulations: injections and dissections

Insect injections were carried out as follows. *Manduca sexta* larvae were anaesthetised and immobilised by placing them on, and covering them in, ice for 10-15 minutes. They were surface sterilised with 70% ethanol and injected by puncturing the hindmost segment of the larva (anterior to the horn) with a disposable 30-gauge hyperdermic needle (BD Microlance) coupled to a 1 ml polycarbonate syringe (BD Diagnostic Systems), and releasing the solution into the hemocoel. The injection volume was in all cases 50 µl.

In order to dissect *M. sexta* larvae the insects were first anaesthetised, immobilised and sterilised as described above. In order to collect larval hemolymph the dorsal horn was cut at the midpoint using clean dissection scissors and bled into individual pre-chilled 1.5 ml

microcentrifuge tubes. To separate hemocytes from the hemolymph plasma the samples were centrifuged at 1,000g for 8 minutes at 4ºC unless otherwise stated. To dissect tissue a cut was made directly to one side of the dorsal horn, after which the insect was opened up by cutting dorsally from the dorsal horn to just behind the head and pinned out. Fat body tissue was removed with forceps. To dissect midgut tissue a 2 mm by 2 mm square section of gut was cut from the dorsal side of the midgut, at the midpoint of the midgut (neither towards the anterior or posterior of the midgut).

In *Blattella germanica* all dissections and treatments were carried out on specimens anesthetized with CO_2. Fat bodies used to determine mRNA levels were dissected out by standard surgical methods, taking care to obtain the same proportion of tissue per specimen each time (ca. 90% of the abdominal fat body). Injections were carried out using a Hamilton syringe.

2.3 Bacterial culture

Sterile technique was observed at all times when culturing bacteria. Bacteria were stored at -80ºC in a 20% glycerol stock and cultured in Luria-Bertani (LB) media, which was made by combining 1L distilled water, 10g Sodium Chloride (Sigma-Aldrich), 10g Tryptone (BD Diagnostic Systems) and 5g of Yeast Extract (Sigma-Aldrich) in a duran bottle, and sterilising the resulting solution by subjecting it to high pressure saturated steam in an autoclave. To make solid agar plates 15 ml agar (BD Diagnostic Systems) was included along with the other components of the LB mixture. After autoclaving the agar mixture it was cooled to approximately 50ºC and poured into 90 mm tissue culture dishes (Sarstedt).

Escherichia coli strain DH5α was prepared for injection by inoculating 5 ml LB liquid media with 5 µl of a 20% glycerol stock culture of *Escherichia coli* and then incubating overnight (for approximately 16 hours) in a shaking incubator at 37ºC and ~220 rpm. The density of the bacterial culture was determined using spectrophotometry at 600 nm, the bacterial cells were washed with sterile phosphate buffered saline (PBS), and diluted to a concentration of 2000 colony forming units (CFU)/ml. For bacterial challenge experiments, insects were injected with 50 µl of this dilution and each insect therefore received a dose of approximately $1x10^5$ CFU. The number of injected bacterial cells was confirmed by making serial dilutions of the injection solution and plating the dilutions onto 1.5% agar plates, followed by incubation at 37°C overnight.

2.4 RNA extraction

Total RNA was isolated from dissected *M. sexta* tissue using a phenol-chloroform extraction protocol. TRI reagent (Sigma-Aldrich), which is a version of the single-step total RNA isolation reagent developed by Chomczynski & Sacchi (1987) and which combines phenol and guanidine thiocyanate in a mono-phase solution to facilitate the inhibition of RNase activity, was used. A volume of TRI reagent (250 µl) was added to the tissue samples in their 1.5 ml microcentrifuge tubes. Tissue homogenisation was achieved by grinding the fat body and midgut by hand with an RNase-free pellet pestle (Anachem) and by repeatedly drawing the hemocyte suspension through a disposable 30-gauge hyperdermic needle (BD Microlance) coupled to a 1 ml polycarbonate syringe (BD Diagnostic Systems). Cellular debris was removed from the resulting homogenates by centrifugation at 12,000 g for 10 minutes at 4°C.

50 µl chloroform (0.2 ml chloroform per of 1 ml TRI reagent) was added to the samples, which were vortexed for 45 seconds, incubated at room temperature for 15 minutes and centrifuged at 12,000 g for 15 minutes at 4°C. The addition of chloroform separated the homogenate into aqueous and organic phases: the upper colourless aqueous phase, containing the RNA, but no DNA or protein, was removed to a new microcentrifuge tube. 125 µl isopropanol (0.5 ml isopropanol per of 1 ml TRI reagent) was added to precipitate the RNA; the samples were incubated for 10 minutes at room temperature before centrifugation at 12,000 g for 10 minutes at 4°C to pellet the RNA. After careful removal of the isopropanol by pipetting, the RNA pellet was washed by adding 500 µl 70% ethanol and centrifuging at 7,500 g for 10 minutes at 4°C. The ethanol was removed by pipetting and the RNA pellet air dried for 5-10 minutes, before being re-suspended in 30 µl diethylpyrocarbonate (DEPC)-treated water (Ambion). RNA samples were stored at -80°C.

Total RNA was isolated from *B. germanica* tissue using a GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Dissected tissue was placed in 1.5 ml microcentrifuge tubes and submerged in liquid nitrogen to "snap freeze" the samples. 250 µl lysis solution containing 1% 2-mercaptoethanol was added to the tissue samples and they were homogenised using a tissue grinder. The homogenised samples were spun through a filtration column to remove cellular debris and shear DNA and then applied to a high capacity silica column to bind total RNA, followed by washing and elution.

2.5 DNase treatment

Total RNA from *M. sexta* tissue was treated as follows. A 15 µl aliquot of RNA solution was transferred to a 96 well microplate. Any contaminating DNA was removed by treating with DNase: 1 µl (2 units) RNase free DNaseI (Ambion, AM2224) and 1.5 µl 10x DNaseI buffer (Ambion, AM8170G) was added to the RNA solution, which was incubated in a thermal cycler (MJ Research) for 1 hour at 37°C. The enzyme was subsequently inactivated by incubation for 10 minutes at 75°C.

RNA preparations from *B. germanica* were treated as follows. 500 ng of each RNA preparation was incubated with 1 µl RNase-Free DNase (Promega, M6101) and 1.15 µl 10x RNase-Free DNase in a total volume of 11.5 µl at 37°C for 30 minutes. The enzyme was subsequently inactivated by incubation for 5 minutes at 75°C.

2.6 Polymerase chain reaction (PCR)

GoTaq® Flexi DNA Polymerase (Promega) was used for all PCRs unless otherwise stated. Reagents were thawed on ice and reactions were set up in 0.2 ml thin-walled PCR tubes in a total volume of 50 µl. A typical reaction contained 0.25 µl (1.25 units) GoTaq Flexi DNA Polymerase, 10 µl GoTaq Flexi 5X Buffer, 4 µl magnesium chloride (final concentration 2 mM), 2 µl dNTPs (final concentration 200 µM) and 15 *p*mol each of the forward and reverse primers. Oligonucleotides (primers) were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/) and OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html). Primers were chosen according to the following design parameters. The melting temperatures of the oligos were between 57°C and 63°C, the size of the primers was between 18 and 27 bases and the GC content was between 20-80%. Oligos were synthesised by MWG Biotech. 1-20 ng complementary DNA (cDNA) or genomic DNA was added to the above reaction mix. A negative (no-template) control, containing the PCR reagents but no DNA, was set up alongside the reactions.

Following set-up of the PCR reactions they were placed in an MJ Research thermal cycler with a heated lid. The typical thermal profile for amplification was as follows: an initial denaturation step of 95°C for 10 minutes, followed by 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute with a final extension step of 72°C for 10 minutes.

PCR products were purified for downstream applications using a Montage PCR centrifugal filter device (Millipore). The products of amplification were applied to regenerated cellulose

membranes in spin column to filter out the primers and unincorporated nucleotides and dry out the samples, following which the samples were reconstituted in water and recovered by an inverted spin.

2.7 Agarose gel electrophoresis

In order to check the integrity of the RNA extractions, and also the size of PCR products agarose gel electrophoresis was used. Typically a 1% agarose gel was used (although when shorter DNA molecules needed to be resolved higher percentage gels were used) and it was made by weighing 0.7 g UltraPure™ Agarose (Invitrogen; Cat. No. 16500100) in a 100 ml conical flask and adding 70 ml of Tris-acetate-EDTA (TAE) buffer (including 242 g of Tris-Base (Sigma-Aldrich), 57.1 ml of Glacial Acetic Acid (Promega), 100 ml 0.5 M EDTA (Sigma-Aldrich), distilled water up to 1000 ml). The mixture was heated in a microwave oven to dissolve the powder. After the solution had cooled to approximately 55°C (after about ten minutes), 1 µl of ethidium bromide (Promega; final concentration 0.5 µg/ml) was added and mixed into the agarose solution by swirling the conical flask. The solution was then poured into a gel block and a gel comb inserted. After the gel had set (after approximately 30 minutes) and the comb removed the gel was placed in a gel tank (BioRad) containing TAE buffer. Samples were mixed with loading buffer (New England Biolabs, composition: 2.5% Ficoll-400 11 mM EDTA 3.3 mM Tris-HCL (pH 8.0) 0.017% SDS 0.015% bromophenol blue) and loaded into the gel (in a total volume of between 5-10 µl). Appropriate DNA markers were run alongside the samples. Typically a 100 bp (100 bp-1,517 bp) or 2-log ladder (0.1–10.0 kb) was used (New England Biolabs). The gel was run at 100V for 60 minutes and was then observed under a UV transilluminator (Alpha Laboratories).

2.8 Cloning of PCR products

In order to calculate the appropriate volume of PCR product to use in the cloning reaction (to achieve the chosen insert:vector molar ratio) the product was first quantified. An aliquot of PCR product was run on an agarose gel with the appropriate marker (as described above). Comparison of the intensity of the band with the intensity of the bands in the DNA marker (containing known quantities of DNA) allowed an estimate of the quantity of dsRNA to be made. Care was taken to run a similar quantity of DNA on the gel as present in the DNA

marker in order to be able to make a sensible comparison (different dilutions of the PCR product were often run to achieve a workable comparison).

PCR products were cloned into the pCR®II-TOPO® vector (Invitrogen). Some DNA polymerase and Taq enzymes have terminal transferase activity and add a 3' nucleotide tail consisting of adenine bases to the amplified PCR product. This enables ligation of the PCR product to the TOPO-TA vector, which contains thymine overhangs. If the enzyme used for amplification does not have terminal transferase activity {for example, in the case of Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen)} then GoTaq® Flexi DNA Polymerase (Promega), which does have terminal transferase activity, was used to add a 3' adenine tail. 30.8 µl of the PCR template was incubated with 10 µl GoTaq Flexi 5x Buffer, 4 µl magnesium chloride (final concentration 2mM), 5 µl dNTPs (final concentration 200 µM) and 0.2 µl (1 unit) GoTaq Flexi DNA Polymerase at 72°C for 20 minutes.

To clone the PCR product 1 ng of vector (0.1 µl) was mixed with 0.33 µl salt solution and 1.57 µl of insert (PCR product). The concentration of insert was adjusted to give a 1:3 insert:vector molar ratio, which had previously been found to be an effective ratio for this vector (C. Fleury, personal communication, 2009). The cloning reaction was incubated for 30 minutes at room temperature to allow ligation to occur, after which it was de-salted in 1% agarose and 100 mM glucose. 0.1 g agarose was melted in 9 ml sterile distilled water in a 15 ml falcon tube (Sarstedt) in a microwave oven. 1 ml 1M glucose was added and mixed once the agarose had dissolved. 1 ml of the resulting solution was poured into 1.5 ml microcentrifuge tubes and an indentation made in the agar by placing a pipette tip through the lid of the tube and into the solution. The agar was allowed to set for approximately 10 minutes on ice and the pipette tip removed. After the cloning reaction had proceeded for 30 minutes 18 µl sterile distilled water was added to the 2 µl reaction, mixed and the resulting 20 µl transferred to the well in the agarose made by the pipette tip. The tube was incubated on ice for 90 minutes to allow de-salting to proceed.

Electrocompetent *Escherichia coli* strain DH5α cells were used for transformations. The cells were stored in 1.5 ml microcentrifuge tubes containing 50 µl aliquots at -80°C: aliquots were removed from the freezer and placed onto ice prior to use. 10 µl of the cloning mix was added to a 50 µl vial of cells and mixed gently by pipetting. After incubation on ice for 45 seconds the mixture was added to an electroporation cuvette (Invitrogen), which had been pre-chilled on ice for 1 hour. The cuvette was tapped on a surface to remove bubbles, and the outside of the cuvette dried with tissue paper. The cuvette was then placed in a cell porator (Life-Technologies) and pulsed (electroporated) at the appropriate voltage to achieve 330 µF capacitance. 500 µl LB containing 20 mM glucose was then added to the cuvette, the

solution was pipetted up and down and removed to a 15 ml falcon tube (Sarstedt), which was incubated in a shaking incubator at 37^{o}C and ~220 rpm for 1 hour. Agar plates were prepared for blue-white screening. 50 µl of 50 mg/ml X-Gal and 100 µl of 0.1 M IPTG was spread onto previously prepared 100 mM ampicillin LB agar plates; the plates were allowed to absorb these additions for 30 minutes. The 500 µl culture was spread onto the prepared LB ampicillin/X-Gal/IPTG plates and incubated overnight (16 hours) at 37°C.

Since X-Gal/IPTG plates had been used for the plating of the transformed bacteria, colonies containing successful ligations could be identified. Pipette tips were used to pick white colonies (containing plasmids with inserts) from the agar plates (10-15 colonies were chosen per plate). The tips were placed into a 1.5 ml microcentrifuge tubes containing 5 µl LB liquid media (these liquid media samples were later plated onto LB plates, annotated and incubated at 37°C to act as a store for the colonies). Colony PCR was used to further screen the colonies. PCR reactions were performed largely as described in section 2.6. The reaction mix (50 µl) was prepared in a 0.2 ml thin-walled PCR tube and forward and reverse M13 primers were used (M13-FP; 5'-TGT AAA ACG ACG GCC AGT-3' & M13-RP: 5'-CAG GAA ACA GCT ATG ACC-3'). M13 sites flank the cloning site in the pCR®II-TOPO® vector; therefore PCR using M13 primers can be used to identify clones containing vectors with inserts of the appropriate size. The pipette tips used to pick colonies were removed from the microcentrifuge tubes and deposited into the PCR reagents for several seconds. The bacterial cells from the colonies were, therefore, used as the template in the PCR reaction. Following thermal cycling as described in section 2.6 the samples were analysed by gel electrophoresis (section 2.7). Colonies containing inserts of the appropriate size (taken from the colony storage plate) were inoculated into 5 ml liquid LB media and grown overnight (for 16 hours) at 37°C. Plasmid DNA was recovered from the overnight cultures using a QIAprep Spin Miniprep Kit (Qiagen).

2.9 DNA sequencing and sequence manipulation

Samples were sent away to GATC Biotech for sequencing. Sanger sequencing was performed using ABI 3730xl technology. Single run (Run24) reactions were performed giving Phred20 quality sequencing {Phred scores are widely used to characterize the quality of DNA sequence; a Phred20 score means that the chance that any base is incorrectly assigned is 1 in 100 (Ewing & Green, 1998; Ewing *et al.*, 1998)}.

Sequence data were generally analysed and processed in Microsoft Word. BLAST (Basic Local Alignment Search Tool; found at http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches,

which use an algorithm to compare a query sequence with a database of sequences, and identify library sequences that resemble the query sequence above a certain threshold, were used to investigate or confirm the identity of cloned sequences. ClustalW2, a general purpose multiple sequence alignment program (found at http://www.ebi.ac.uk/Tools/msa/clustalw2/), was used to align sequences of interest. Editing and annotating multiple sequence alignments took place using GeneDoc (Nicholas & Nicholas, 1997). Phylogenetic analysis was performed using the neighbour-joining method of Saitou & Nei (1987) and phylogram trees were rooted using NJplot (Perrière & Gouy, 1996). Conserved domains were identified using the ExPASy ScanProsite conserved domain search (http://expasy.org/tools/scanprosite/). In many cases sequence data generated were manipulated further using various tools found at http://expasy.org/tools/.

2.10 Quantitative reverse transcription PCR (q-RT-PCR)

A two-step quantitative reverse transcription PCR (q-RT-PCR) protocol was used to quantitate transcript levels in insect tissue. qRT-PCR was performed in *M. sexta* as follows. First, the concentration of the total RNA samples (obtained as described in section 2.4 and treated with DNase as described in section 2.5) was ascertained so that equal quantities of RNA could be used for complementary DNA (cDNA) synthesis. Aliquots of RNA were diluted and quantified with a Qubit® 2.0 Fluorometer (Invitrogen) which works by detecting signals emitted by fluorescent dyes selective for specific targets (in this case the dye is specific for RNA). 10 µl of each RNA sample was added to 190 µl of a working solution of reagents from the Qubit™ RNA Assay Kit (Invitrogen, Q32852; the working solution was made by diluting the Qubit™ RNA reagent 1:200 in Qubit™ RNA buffer) in Qubit assay tubes (Invitrogen, Q32856). The solution was mixed by vortexing for 2-3 seconds and incubated at room temperature for 2 minutes, after which a fluorescence reading was taken on the Fluorometer. The concentration of the RNA was calculated from the reading, taking into account the dilutions of the original RNA solution.

Complementary DNA (cDNA) synthesis was performed using 125 ng total RNA. In the first step of the reverse transcription (RT) reaction RNA was mixed with 125 ng random hexadeoxynucleotides (primers; Promega, C1181) in a total volume of 8 µl DEPC-treated water (Mobio Laboratories) in a 96 well microplate (Greiner Bio-One). The solutions were mixed by pipetting and the plate spun briefly, after which the samples were heated to 75°C for 10 minutes in a thermal cycler (MJ Research) to denature RNA secondary structure. The plate was quickly chilled on ice. A master mix of MMLT reverse transcriptase (Promega; 0.5 µl per RNA sample), 5x MMLT reverse transcriptase buffer (Promega; 2.5 µl per RNA

sample), dNTPs (Promega; 1.25 µl per RNA sample; concentration 5 mM) and RNase inhibitor (Promega; 0.30 µl per RNA sample) was prepared on ice and added to the RNA samples. The samples were mixed by pipetting, spun briefly, and then incubated at 37°C for 60 minutes in a thermal cycler (MJ Research), after which the enzyme was deactivated by heating to 70°C for 15 minutes. The cDNA samples were stored at -20°C.

A comparative C_T (ΔC_T) method of qPCR was employed, where the Ct value (threshold value) of one target gene was compared to the Ct value of another internal control gene, in a single sample. In order for this method to be valid the efficiency of the target amplification (the gene of interest) and the efficiency of the reference amplification (the internal control gene) must be approximately equal. Care was therefore taken to ensure that the efficiency of all primer pairs was close to 100%. One precaution taken to ensure equally efficient amplification was to design the primers so that they all produced an amplicon of approximately 100 base pairs (bps). Deficient binding of the primers to the template sequence because of secondary structures could result in inefficient amplification. Consequently, the folding of the template DNA sequence and the formation of internal hairpins at the annealing temperature of 60°C was predicted using mfold software (found at http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form) and only primers located in regions of the sequence lacking hairpin loops were selected for further analysis. To confirm the efficiency of the primer pairs standard curve analysis of a dilution series was performed and efficiencies in the range of 98-102% were considered acceptable. Dissociation curve analysis was also performed for each primer pair to ensure all samples yielded a single sharp peak at the amplicon's melting temperature.

Real-time PCR was carried out using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) and iTaq SYBR Green Supermix (Bio-Rad) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye. PCR reactions were carried out in duplicate using 7.5 *p*mol specific primers and approximately 5 ng cDNA (equivalent of 5 ng RNA in RT reaction) in a total volume of 15 µl. Ribosomal protein S3 (rpS-3) was used as the internal control gene. The thermal profile for amplification was as follows: 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. The primers used to amplify *M. sexta* genes are detailed in Table 2.1. All the primer sequences were designed in our laboratory (by either C. Fleury or by me) except those used to amplify cecropin, which were designed by An *et al.* (2009).

qRT-PCR was performed in *B. germanica* using a similar protocol. The concentration of the total RNA samples (obtained as described in section 2.4 and treated with DNAse as described in section 2.5) was determined using a Nanodrop Spectrophotometer

(ThermoScientific). Complementary DNA (cDNA) synthesis was performed using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). In the first step of the reverse transcription (RT) reaction 10 µl RNA (500 ng) was mixed with 1 µl Oligo d(T) (final concentration 2.5 µM) and 2 µl random hexadeoxynucleotides (final concentration 60 µM) in a 0.2 ml thin-walled PCR tube. The solutions were mixed by pipetting and spun briefly, after which the samples were heated to 65°C for 10 minutes in a water bath to denature RNA secondary structure. The samples were quickly chilled on ice to allow the primers to anneal to the RNA. A master mix of reverse transcriptase (0.5 µl per RNA sample, 10 U), reverse transcriptase buffer (4 µl per RNA sample), dNTPs (2 µl per RNA sample, final concentration 1 mM) and Protector RNase Inhibitor (0.5 µl per RNA sample, 20 U) was prepared on ice and added to the RNA samples. The samples were mixed by pipetting, spun briefly, and then incubated at 25°C for 10 minutes then 55°C for 60 minutes in a water bath, after which the enzyme was deactivated by heating to 85°C for 5 minutes. The cDNA samples were stored at -20°C.

A comparative C_T method (ΔC_T) was also employed to quantify gene expression in *B. germanica.* qPCR was performed using iTaq SYBR Green Supermix (Bio-Rad) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye. PCR reactions were carried out in triplicate using 6 *p*mol specific primers and approximately 5 ng cDNA (equivalent of 5 ng RNA in RT reaction) in a total volume of 20 µl. Actin 5C was used as the internal control gene. The primers used to amplify *B. germanica* genes are detailed in Table 2.1. The thermal profile for amplification was as follows: 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds then one cycle of 95°C for 1 minute, 60°C for 1 minute and 55°C for 30 seconds.

Table 2.1: Primer sequences used in q-RT-PCR experiments.

Gene	Accession number	Primer name	Primer Sequence 5'-> 3'	Amplicon length (nt)
Manduca sexta				
ribosomal protein S3 (RpS3)	P48153	Ms_rpS-3_qPCR_F Ms_rpS3_qPCR_R	CATGATCCACTCCGGTGAC GACCTTAATTCCGAGCACTCC	90
attacin 2	AY232304	Ms_attacin2_qPCR_F Ms_attacin2_qPCR_R	TATCGGTGGGGCTGATTTTA ATGTGGGTATCCGTCAAACTG	108
cecropin	AY232302	Ms_cecropin_qPCR_F Ms_cecropin_qPCR_R	CCGTGTTTTATTCTTCGTCTTC AATCCTTTGACCTGCACCC	103
gloverin	AY232303	Ms_gloverin_qPCR_F Ms_gloverin_qPCR_R	CCCGCAATACGCTCAGATA ATTGTTGCCGACTTGCTTGT	96
moricin	AY232301	Ms_moricin_qPCR_F Ms_moricin_qPCR_R	GCGTTGTCGCTATTATTTTCG TTGCTCGTAGACCTTTTCCAA	103
hemolin	U11879	Ms_hemolin_qPCR_F Ms_hemolin_qPCR_R	GAAGTCCTCTTCCGGGAGTC CTGGGCGATATTGTGTTCCT	129
immulectin-2 (IML-2)	AF242202	Ms_IML2_qPCR_F Ms_IML2_qPCR_R	GCCGAAGGTGGATACTTGAC ACCATAGACGCTGGAAGGTG	90
serine protease homologue 3 (SPH-3)	AF413067.1	SPH3_qPCR_F SPH3_qPCR_R1	ACCGCAGTTTAAGGGAAGGA CAAACAGATAGGGCGGATGT	91
dicer-2		Ms_dicer2_qPCR_F2 Ms_dicer2_qPCR_R2	TGAAGGCGAGACCATATCAA ACGTTTTCCCAGACCCTGT	90
argonaute-2		Ms_ago2_qPCR_F2 Ms_ago2_qPCR_R2	ACCACCTCGTGTTGTATAGGAAG CCTGTGAAAACTGTCCCTCTG	93
Non-insect				
GFP		eGFP_qPCR_F eGFP_qPCR_R	CCGACCACTACCAGCAGAAC TTGGGGTCTTTGCTCAGG	100
Blattella germanica				
actin 5C	AJ862721.1	BgActin5C_F BgActin5C_R	AGCTTCCTGATGGTCAGGTGA TGTCGGCAATTCCAGGGTACATGGT	213
vitellogenin	AJ005115.2	VgRt488Fw VgRt373Rv	CTGGGCATTTGACAACACAACAT TTGAAGAGCTGCTGGAGAGTTTG	116

2.11 dsRNA synthesis

Double-stranded RNA (dsRNA) was prepared for injection into *M. sexta* using PCR and *in vitro* transcription as described in Clemens *et al.* (2000) and illustrated in Figure 2.1. The first step was the amplification of the target DNA sequence using PCR with specific forward and reverse primers that included terminal 5' T7 promoter sites (sequence 5'-TAATACGACTCACTATAGGGAGA-3': Figure 2.1a). The PCR reagents and the thermal profile used were exactly as described in section 2.6 and the primers used are detailed in Table 2.2. The template for the PCR was typically cDNA prepared from *M. sexta* larvae as

described in section 2.10. In the case of dsRNA for eGFP a TOPO plasmid containing the eGFP sequence (PCRII_Topo_eGFP) was used as a template (the plasmid was kindly provided by Ananya Jeshtadi of Oxford Brookes University). Following purification of the PCR product using a Montage PCR centrifugal filter device (Millipore), the purified product was used as a template in a transcription reaction. A T7 MEGAscript® High Yield Transcription Kit (Ambion) was used to simultaneously transcribe two complementary RNA strands from the template following the manufacturer's instructions except that reactions were allowed to proceed overnight (10–16 h) to increase RNA yield (Figure 2.1b). Because the two single-stranded RNA molecules synthesised were complementary they spontaneously annealed to each other (Figure 2.1c)

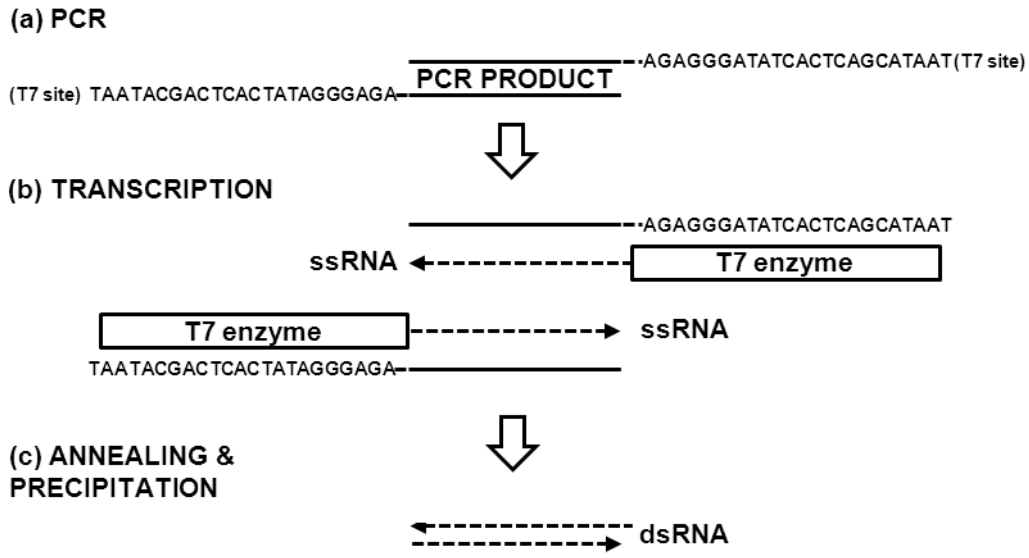


Figure 2.1: dsRNA preparation for injection into *M. sexta* (a) PCR was used to generate amplicons with 5' flanking T7 sites. **(b)** Transcription of the amplicons generated two complementary single-stranded RNA molecules. **(c)** The single-stranded RNAs spontaneously annealed to form double-stranded RNA (dsRNA).

In order to destroy any remaining DNA template 1 µl TURBO DNase (provided in the MEGAscript® kit) was added to the dsRNA sample, which was then incubated for 15 min at 37°C in a water bath. The dsRNA product was purified by lithium chloride precipitation. 30 µl nuclease-free water and 30 µl LiCl Precipitation Solution (both provided in the kit) were added to the dsRNA, the samples were mixed by pipetting and incubated at -20°C for 30 minutes. Following incubation the RNA was collected as a pellet by centrifugation at 4°C

for 15 min at 12,000 g. The supernatant was carefully removed and the pellet washed with 1 ml 70% ethanol and centrifuged at 4°C for 15 min at 12,000 g. The 70% ethanol supernatant was carefully removed and the RNA pellet resuspended in 30 µl nuclease-free water (from the MEGAscript® kit). The concentration of the RNA was determined using spectrophotometry. The dsRNA product was diluted 100 fold in a UVette® (Eppendorf), placed in an Eppendorf BioPhotometer and a reading at OD260 was taken. Multiplying the OD260 by the dilution factor and an extinction coefficient of 45 gives the concentration of dsRNA in µg/ml.

Table 2.2: Primer sequences used to synthesise *M. sexta* and GFP dsRNAs. The T7 promoter sequence is shown in bold.

Gene	Primer name	Primer Sequence 5'-> 3'	Length (bps)
GFP	eGFP_T7_F	**TAATACGACTCACTATAGGGAGA**CCTGAAGTTCATCTGCACCA	541
	eGFP_T7_r	**TAATACGACTCACTATAGGGAGA**GAACTCCAGCAGGACCATGT	
moricin	Moricin_T7_F3	**TAATACGACTCACTATAGGGAGA**ATATTGCTGGCACCACACAC	216
	Moricin_T7_R3	**TAATACGACTCACTATAGGGAGA**AAGATTCCGAAGGGAGAACG	
insecticyanin A	InsA_T7_F	**TAATACGACTCACTATAGGGAGA**CTCGACTCTCCTTCGACACC	869
	InsA_T7_R	**TAATACGACTCACTATAGGGAGA**GAAATCGCGAAGGCATTTT	
insecticyanin B	InsB_T7_F	**TAATACGACTCACTATAGGGAGA**TCTGTTGTTCTGAGCCACAAAT	569
	InsB_T7_R	**TAATACGACTCACTATAGGGAGA**CACAGTGTGCTTTCTGTTTTCTG	
hemolin	Ms_hemolin_T7_f	**TAATACGACTCACTATAGGGAGA**ACGGCAAAGAATTCAAATGG	613
	Ms_hemolin_T7_r	**TAATACGACTCACTATAGGGAGA**TTGAACCAGTTGGGGTAAGC	
immulectin-2 (IML-2)	Ms_IML2_T7_F1	**TAATACGACTCACTATAGGGAGA**GACTCTTGCGAGTCGTGTGA	953
	Ms_IML2_T7_R1	**TAATACGACTCACTATAGGGAGA**GACTGTTTGGGTCCTTTTCG	
nitric oxide synthase (NOS)	NO T7 F2	**TAATACGACTCACTATAGGGAGA**CCAAGACGCTCAGACTTTCC	1045
	NO T7 R2	**TAATACGACTCACTATAGGGAGA**GCGGGCTCCTGATAATCATA	
serine protease homologue 3 (SPH-3)	SPH3_T7_F	**TAATACGACTCACTATAGGGAGA**CGTGGCACGATAATGTTGT	657
	SPH3_T7_R	**TAATACGACTCACTATAGGGAGA**AGTCGCTGCGTCAATGTATG	
vATPase subunit A	MS_VATPase_A_F2	**TAATACGACTCACTATAGGGAGA**GTTTGAAGACGATCGCCAAT	822
	MS_VATPase_A_R2	**TAATACGACTCACTATAGGGAGA**GCAACCGACGTAGATGATGA	
vATPase subunit C	MS_VATPase_c_F2	**TAATACGACTCACTATAGGGAGA**ACGGACCCTTCTTTGGAGTT	425
	MS_VATPase_c_R2	**TAATACGACTCACTATAGGGAGA**CGACGATGAGACCGTACAGA	
vATPase subunit G	MS_VATPase_G_F2	**TAATACGACTCACTATAGGGAGA**CAGAAATGGCGAGTCAGACA	353
	MS_VATPase_G_R2	**TAATACGACTCACTATAGGGAGA**CTACGCGGTAGTTGATGTGC	

Double-stranded RNA (dsRNA) was prepared for injection into *B. germanica* as described by Martín *et al.* (2006) and illustrated in Figure 2.2. In brief PCR fragments were cloned into the pSTblue-1 vector (Novagen). For *vitellogenin* (dsBgVg) a 732 bp fragment from amino acid 746–990 (GenBank accession number: CAA06379) was cloned. A control dsRNA,

consisting of 92 bp of non-coding sequence from the pSTblue-1 vector, was also produced. To synthesise RNA the plasmid was first digested with Sal1 and BamH1 enzymes (Promega) overnight at 37°C (separate reaction – one reaction for each enzyme). The two linearised plasmids were precipitated with phenol chloroform and then used as templates for single-stranded RNA synthesis. *In vitro* transcription was performed using T7 or SP6 RNA polymerase (T7 for Sal1 digestion and SP6 for BamH1 digestion). To prepare dsRNA, equimolar amounts of sense and antisense RNAs were mixed, heated for 5 min at 90°C, cooled down slowly to room temperature and stored at -20°C until use. dsRNAs were suspended in diethyl pyrocarbonate-treated water and dissolved in Ringer saline with a final concentration of 1 µg/µl. Formation of dsRNAs was confirmed by running 1 µl on a 1% agarose gel.

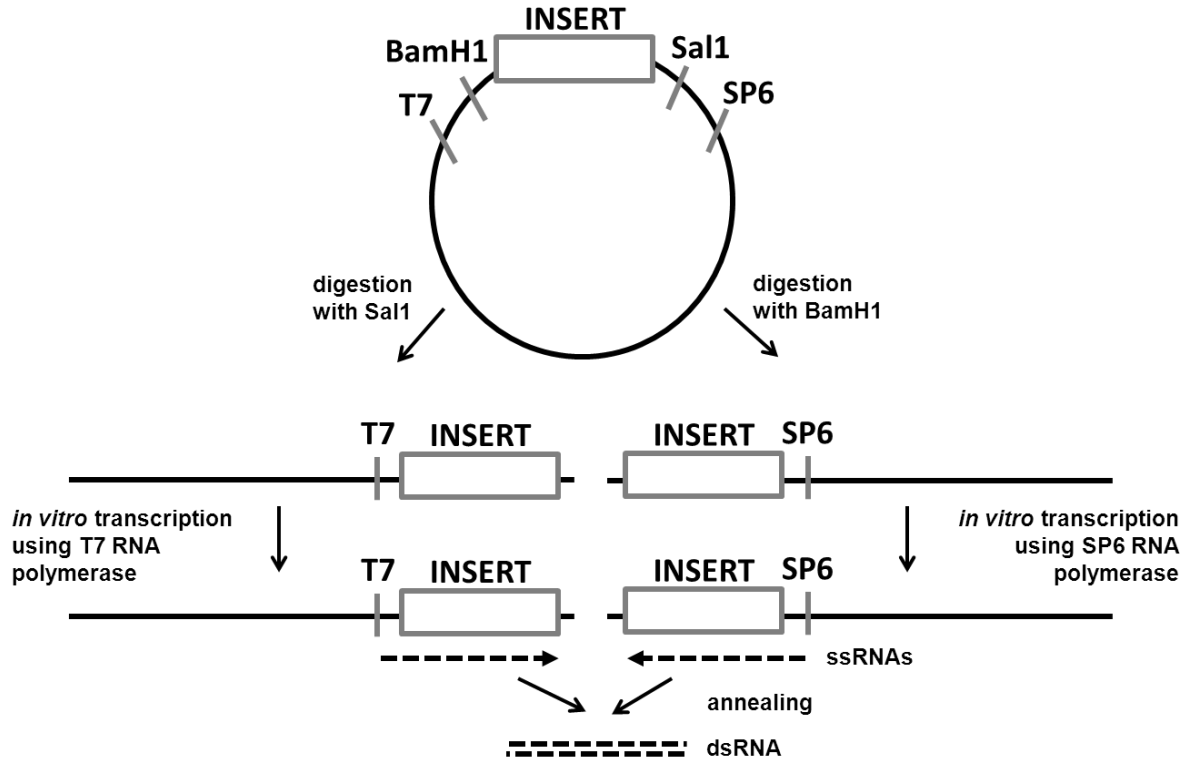


Figure 2.2: dsRNA preparation for injection into *B. germanica*. The plasmid containing the target sequence was digested in separate reactions with Sal1 and BamH1 restriction enzymes. Single-stranded RNAs (ssRNAs) were synthesised using either T7 or SP6 RNA polymerase and annealed by mixing equal quantities of each RNA strand, heating to 90°C and slowly cooling to room temperature.

CHAPTER 3: RNA INTERFERENCE IN TWO INSECT SPECIES: *MANDUCA SEXTA* (LEPIDOPTERA) AND *BLATTELLA GERMANICA* (BLATTODEA)

3.1 Introduction

RNA interference (RNAi) is an endogenous gene silencing mechanism triggered by double-stranded RNA molecules (dsRNAs; Fire *et al.*, 1998), which has proved useful in elucidating gene function in insects. The utility of RNAi as a reverse genetics tool has led to great interest within the insect research community in using RNAi and RNAi experiments have been attempted in many insect species (Mito *et al.*, 2011). While successful knockdowns have been achieved in many cases (for example Magalhaes *et al.*, 2008; Xi *et al.*, 2008; Moriyama *et al.*, 2008; Miller *et al.*, 2008; Marco Antonio *et al.*, 2008; Chen *et al.*, 2008; Bautista *et al.*, 2009; Lemke & Schmidt-Ott, 2009; Lee *et al.*, 2009; Tsao *et al.*, 2009; Walshe *et al.*, 2009; Angelini *et al.*, 2009; Lu *et al.*, 2009; Moczek & Rose, 2009; Sim & Denlinger, 2009; Eleftherianos *et al.*, 2009*a*), the efficacy of RNAi in insects is by no means universal and many species or groups of species are considered to be insensitive to RNAi (Terenius *et al.*, 2011).

This thesis explores potential explanations for the insensitivity of insects to RNAi (including deficiency in the survival of dsRNA in the hemolymph, the uptake of dsRNA into tissue, the spread of the RNAi signal and the expression of the RNAi machinery). Two insect species were used in this study. The first is the tobacco hornworm, *Manduca sexta* (Sphingidae: Lepidoptera) which is widely used as a model organism to study (*inter alia*) the biochemical basis of physiological processes (Zou *et al.*, 2008), the nervous system and its control of behaviour (Raguso *et al.*, 2005), growth and metabolism (Gibellato & Chamberlin, 1994) and the immune system (Kanost *et al.*, 2004). Its large size and rapid generation time and the ease with which it can be reared on a wheat germ based diet have contributed to the utility of this insect as an insect model (Kanost *et al.*, 2004; Raguso *et al.*, 2005). The second insect used in this study is the German cockroach, *Blattella germanica* (Blattellidae: Blattodea), which has been used as a model of the hemimetabolan mode of metamorphosis (Martín *et al.*, 2006; Mané-Padrós *et al.*, 2008; Cruz *et al.*, 2008), as well as in the study of sex pheromone biosynthesis (Tillman *et al.*, 1999), insecticide resistance (Scharf *et al.*, 1996) and circadian rhythm control (Lin *et al.*, 2002; Wen & Lee, 2008; Lee *et al.*, 2009).

Successful RNAi experiments have been published using both *Manduca sexta* (Levin *et al.*, 2005; Eleftherianos *et al.*, 2006*b*, 2006*a*, 2007*a*, 2007*b*, 2009*b*, 2009*a*; Zhuang *et al.*, 2007*a*,

2007*b*, 2008; Soberón *et al.*, 2007; Whyard *et al.*, 2009; Cancino-Rodezno *et al.*, 2010) and *Blattella germanica* (Ciudad *et al.*, 2006, 2007, 2007; Martín *et al.*, 2006; Maestro & Bellés, 2006; Cruz *et al.*, 2006, 2008; Mané-Padrós *et al.*, 2008; Revuelta *et al.*, 2009; Suazo *et al.*, 2009; Gomez-Orte & Belles, 2009; Guo *et al.*, 2010; Huang & Lee, 2011). Nevertheless, during my investigations I found that, whilst *B. germanica* was highly sensitive to gene silencing by RNAi, *M. sexta* appeared to be, in general, insensitive to RNA interference.

This chapter includes examples of RNAi experiments in *M. sexta* and *B. germanica* in order to highlight the disparity in their sensitivity to RNAi. Following initial observations that *M. sexta* was not very responsive to RNAi, a number of experiments were conducted in order to ensure that this lack of gene silencing was not caused by the erroneous specification of key parameters in experimental protocols.

3.2 Results and Discussion

3.2.1 RNAi of vitellogenin *in* Blattella germanica

An experiment was conducted in *Blattella germanica* with the aim of the silencing the *vitellogenin* gene, which encodes a protein precursor of Vitellin, the major protein component of egg yolk (Comas *et al.*, 2000). The *B. germanica vitellogenin* gene is expressed in the fat body of female cockroaches; expression begins 24 hours after adult emergence and peaks after five days of adult life (Martín *et al.*, 1998). Synthesised Vitellogenin is released into the hemolymph and is incorporated into developing oocytes, where it is processed into Vitellin (Martín *et al.*, 1995). In an RNAi experiment to knock down the *vitellogenin* gene dsRNA for vitellogenin (dsBgVg) was injected into newly emerged adult females. Five days after injection with *vitellogenin* dsRNA (and at the peak of its expression) *vitellogenin* mRNA levels in fat body were found to be significantly reduced in comparison to the control (Figure 3.1a; t-test of log transformed data; $t=-11.47$, $p<0.001$, $n=3$). *Vitellogenin* transcripts in the fat body of dsBgVg-injected insects were approximately 2000 fold fewer than in fat body from insects injected with a control dsRNA, representing a rather impressive knockdown of gene expression. Furthermore, ovarioles from dsBgVg -injected insects were smaller in size compared to the control (they showed a 34% reduction in length; Figure 3.1b; t-test: $t=6.12$, $p<0.001$, $n=4$), a phenotype previously observed in RNAi knockdowns of *vitellogenin* and concomitant with a reduction in ovary protein content (Martín *et al.*, 2006).

3.2.2 RNAi of moricin in Manduca sexta

RNAi experiments conducted with the aim of suppressing expression of the *Manduca sexta moricin* gene, which codes for an antimicrobial peptide (AMP) with antibacterial activity against several Gram-positive and Gram–negative bacteria (Hara & Yamakawa, 1995), were, in general, unsuccessful. During RNAi knockdown experiments for inducible immune genes, like *moricin,* gene expression was induced by challenge with *E. coli* cells. Newly emerged fifth instar *M. sexta* larvae were injected with dsRNA (suspended in DEPC-treated water) specific to the target gene 6 hours before challenge with *E. coli* {injection of approximately $5x10^5$ colony forming units (CFUs) suspended in phosphate buffered saline; PBS}. Controls included DEPC and an irrelevant dsRNA in the primary injection and PBS without *E. coli* in the secondary injection. After the secondary injections insects were held at 25°C for 18 hours and then dissected to collect tissue (for the experimental scheme see Figure 3.2a).

The result of a typical experiment to silence *moricin* gene expression is shown in Figure 3.2b. *Moricin* transcript levels in fat body were elevated in response to *E. coli* injection (an increase of approximately 6,000 fold). Within the insects treated with *E. coli* cells those insects injected with dsRNA for *moricin* (dsMOR) did not show significantly reduced transcript levels compared with insects injected with water or an irrelevant dsRNA control (ANOVA found no significant effect of secondary injection treatment; $F=0.2786$, $p=0.7661$).

3.2.3 RNAi troubleshooting in M. sexta

Following several unsuccessful attempts to knock down genes using RNAi in *M. sexta,* a systematic approach was employed in order to ensure that suboptimal experimental conditions were not responsible for the ineffective gene silencing in this insect. A number of experiments were conducted during which several key experimental parameters were adjusted. A summary of these troubleshooting experiments is presented in Table 3.1.

3.2.3.1 Dose of dsRNA

The dose of dsRNA delivered was the first parameter under examination. In initial experiments (for example in the experiment presented in Figure 3.2b), the dose of dsRNA employed was 100 ng, which was the dosage used in several previous RNAi studies targeting immune-related genes in *M. sexta* (Eleftherianos *et al.*, 2006*b*, 2006*a*, 2007*a*,

2007*b*, 2009*b*, 2009*a*). It is possible that this dose was not sufficiently large. In fact, many successful studies using RNAi in Lepidoptera have used much larger doses. For instance, Soberón *et al.* (2007), in their experiment targeting a cadherin gene, injected 1 µg dsRNA into *M. sexta* first instar larvae and Rajagopal *et al.* (2002) administered 4 µg dsRNA to fifth instar *Spodoptera litura* larvae in order to silence expression of the midgut *aminopeptidase N* gene. When taking the mass of the test insects into account these dosages are orders of magnitude higher than those employed in our initial experiments. In the case of the Soberón *et al.* (2007) study the researchers injected 1 µg dsRNA into first instar *M. sexta* larvae (one larva weighs approximately 1.5 mg), giving a relative dose of 667 ng dsRNA / mg insect tissue. This dose is much larger than that employed in the initial experiments to target *moricin* presented in this chapter (Figure 3.2) of 0.1 ng dsRNA / mg tissue (the dose of dsRNA was 100 ng and a fifth instar *M. sexta* larva weighs approximately 1 g). In addition to the dose of dsRNA being potentially too small to stimulate a reduction in gene expression it is also possible that the dose of dsRNA delivered may be too large and result in the induction of gene expression, counteracting any reduction in transcript levels achieved. There is some evidence that this can occur; Hirai *et al.* (2004) found that expression of the immune-related protein Hemolin was induced following introduction of dsRNA in the moth *Hyalophora cecropia.*

In order to ensure that a non-optimal dose of dsRNA was not responsible for the lack of effective RNAi in *M. sexta* a number of RNAi experiments were conducted, during which *moricin* was once again targeted. Doses of dsRNA varying from 1 ng to 10 µg were delivered in experiments similar in design to the one depicted in Figure 3.2a. However, in order to save resources during troubleshooting experiments, not all appropriate controls were included (if a dose was found to have been effective in reducing *moricin* transcript levels the experiment would have been repeated with all the relevant controls). Increasing the dose of dsRNA appeared, at first, to be a promising approach, since an experiment where the dose of *moricin* dsRNA was raised to 1 µg resulted in a partial reduction in gene expression (Figure 3.3; Experiment 1). *Moricin* transcript levels were significantly reduced in comparison to the water control (a 13% reduction; t-test; $t=3.102$, $p=0.0146$). However, when this experiment was repeated no reduction in transcript levels was observed in the dsRNA treated insects (Figure 3.3; Experiment 2; t-test; $t=-0.739$, $p=0.4809$). Further experiments varying the dose of dsRNA delivered were not successful in achieving an RNAi knockdown of *moricin* gene expression and it was therefore concluded that an inadequate or inappropriate dose of dsRNA was not responsible for the lack of effective RNAi.

3.2.3.2 Target gene

It is possible that insect genes vary in their degree of susceptibility to RNAi. One study, concerned with the development of an RNAi pesticide, carried out an RNAi screen of 290 genes in the western corn rootworm, *Diabrotica virgifera virgifera*, and found considerable variation in the target genes' susceptibility to RNAi (Baum *et al.*, 2007). Potential explanations for varying susceptibility include that dsRNAs or resulting siRNAs may be degraded in a sequence-specific manner or that certain genes with efficient feedback mechanisms may be able to prevent the depletion of mRNA by increasing their rate of transcription (Terenius *et al.*, 2011). An additional possible explanation arises from the suggestion that RNAi is only capable of targeting the destruction of newly synthesised mRNA (Nishikawa & Natori, 2001). If this were the case then genes with a high mRNA turnover rate might be more susceptible to RNAi. It was for this reason that the inducible immune-related gene *moricin* was targeted during the RNAi troubleshooting process, since challenge with *E. coli* leads to the production of newly transcribed mRNA, which should theoretically be susceptible to targeting by RNAi.

Several other immune-related genes were targeted during the troubleshooting process, including *hemolin*, a pattern recognition protein (PRP) which may be involved in opsonisation or in trapping bacteria in the nodules formed by hemocytes (Kanost *et al.*, 2004; GI:508721); *serine protease homologue 3* (SPH-3), a gene with a poorly understood role in the immune response (Felföldi *et al.*, 2011); *immulectin-2* (IML-2; GI:237863090), which has a role in the activation of phenoloxidase (Kanost *et al.*, 2004); and *nitric oxide synthase* (NOS; GI:3372749), which produces nitric oxide, a diffusible, reactive and membrane-permeable small molecule which may be important in mediating the insect immune defence against oral infection (Eleftherianos *et al.*, 2009*a*). In addition to these immune genes a number of other targets were chosen. Several subunits of a gut vacuolar ATPase (v-ATPase; subunit A GI:11061, subunit C GI:5852161 & subunit G GI:1061194), an ATP-dependent ion pump responsible for ion transport across the midgut plasma membrane in *M. sexta* larvae (Wieczorek *et al.*, 1999; Grüber, 2003), were targeted by delivery of dsRNA by injection and by feeding. The final target was the *insecticyanin* gene (A GI:9715 & B GI:9717), which codes for a blue biliprotein, synthesised in the epidermis of *M. sexta* larvae, which (combined with yellow carotenoids derived from the diet) produces a green hue that provides effective camouflage for the larva (Kawooya *et al.*, 1985; Kang *et al.*, 1995). As detailed in Table 3.1 changing the target gene did not affect the outcome of the RNAi experiments and successful and consistent silencing was not achieved when targeting any of the above genes. Since nine genes, with rather distinct functions, were

targeted during this study it seems unlikely that lack of effective RNAi was due to the particularly refractory nature of the target genes.

3.2.3.3 Tissue

It has been documented that different tissue types can be differentially susceptible to RNAi. For instance, in the nematode worm, *C. elegans*, the gonad and neurons are resistant to RNAi whilst the remainder of the tissue is susceptible to RNAi. The refractory nature of the worm gonad and neurons has been linked to their unusually high expression levels of the *eri-1* (*enhanced RNAi-1*) gene, which encodes a nuclease (Kennedy *et al.*, 2004). Terenius *et al.* (2011) in their review of lepidopteran RNAi found that, in general, epidermal tissue was rather refractory to RNAi, whereas brain tissue was rather sensitive. In the troubleshooting experiments conducted in this study the genes targeted were expressed in a number of different tissues. The immune-related genes *moricin, SPH-3, IML-2 hemolin* and *NOS* are expressed in fat body and hemocytes (Eleftherianos *et al.*, 2006*b*, 2007*b*, 2009*a*), the *vATPase* subunits (*A, C* and *G*) are expressed in midgut tissue (Wieczorek *et al.*, 1999) and the *insecticyanin* gene is expressed in the epidermis (Kang *et al.*, 1995). It is unlikely, therefore, that the difficulties experienced in performing RNAi in *M. sexta* are caused by tissue-specific insensitivity to RNAi.

3.2.3.4 Size of the dsRNA molecule

The size of the dsRNA molecule delivered during RNAi experiments may have an impact on the degree of silencing achieved. It certainly is reasonable to suppose that the length of the dsRNA will affect the outcome of the RNAi experiment, since the silencing effect of long dsRNAs is a result of the pooled effect of the many siRNA molecules produced intracellularly by Dicer-2 (Arziman *et al.*, 2005). These siRNA molecules are likely to vary in their efficacy (caused by variation in several parameters likely to include GC content, asymmetry and thermodynamic stability; Ui-Tei *et al.*, 2004). Therefore, one can predict that longer dsRNA molecules should be more effective, since they are cleaved into more siRNA molecules, increasing the pooled effect of the siRNAs as well as the chance of producing highly effective siRNAs.

There is some evidence for the superior silencing efficiency of longer dsRNA molecules. Feinberg & Hunter (2003) found that long dsRNA (500 bps) silences at much lower molar concentrations than shorter dsRNA {19 bp (siRNA), 50 bp &100 bp}. They ruled out the

possibility that preferential transport of long dsRNA was responsible for this disparity in silencing efficiency (they found that the mass of accumulated 50 bp, 100 bp & 500 bp dsRNA was indistinguishable) and concluded that in *Drosophila melanogaster* S2 cells long dsRNA is simply a more effective RNAi trigger than short dsRNA (Shih *et al.*, 2009). In contrast with these results, another group of researchers, again working with *D. melanogaster* S2 cells, found that shorter dsRNAs (180 bps in length) appear to be as effective as longer dsRNAs (500 bps and 778 bps in length) at inducing RNA interference (Betz, 2003).

It is, therefore, not absolutely clear whether longer dsRNA molecules show greater efficacy as silencing triggers. An additional consideration when designing dsRNA constructs is that longer dsRNA molecules may carry an increased risk of off-target effects (because the production of more siRNAs means that the probability is increased that at least one of them has sequence homology to one or more genes other than the target). So, which size of dsRNA is appropriate? In general, dsRNA used in non-mammalian systems to induce RNAi is more than 400 nucleotides in length, encompassing the majority of the target mRNA sequence (Betz, 2003; for example (Yang *et al.*, 2000). In the experiments presented here dsRNA constructs with a range of lengths were employed (see Table 3.1). Design of the constructs was often constrained by the size of the target transcript, as well as the need to design qPCR primers which lie outside the target region. In the main, constructs were designed to be as large as possible and they varied from 216 to 1054 nucleotides in length. It is unlikely, therefore, that the RNAi experiments conducted did not work because the dsRNA constructs were not the most effective size.

3.2.4 Evidence for the insensitivity of **M. sexta** *to RNAi*

Although there have been several published studies utilising RNAi in *Manduca sexta* (Levin *et al.*, 2005; Eleftherianos *et al.*, 2006*b*, 2006*a*, 2007*a*, 2007*b*, 2009*b*, 2009*a*; Zhuang *et al.*, 2007*a*, 2007*b*, 2008; Soberón *et al.*, 2007; Whyard *et al.*, 2009; Cancino-Rodezno *et al.*, 2010) there is evidence that many researchers have experienced difficulties achieving RNAi in *M. sexta*. Terenius *et al.* (2011), *in their study which* integrated published and unpublished results from RNAi experiments, revealed that of the 33 studies entered into the database a "high" degree of silencing (a subjective measure of silencing as provided by the author) was observed in less than half (42%; 14/33; Figure 3.4). In contrast, RNAi experiments in *Bombyx mori* (the only other insect for which there were more than 30 database entries) resulted in a "high" degree of silencing more than half of the time (59%; 23/39; Figure 3.4). Roughly the same proportion of studies in both insects resulted in no

silencing (*M. sexta* 27%, *B. mori* 28%), whereas more studies in *M. sexta* resulted in a low degree of silencing (13% *B. mori*) (30% *M. sexta*). There is some evidence, then, in additional to the experimental data present here, that *M. sexta* larvae are insensitive or at best variably sensitive to RNAi.

3.3 Methods

3.3.1 B. germanica *RNAi*

Double-stranded RNA (dsRNA) for *vitellogenin* and the non-coding control was prepared as described in Section 2.11. The dsRNA solution was adjusted to a final concentration of 1 µg μl^{-1} in Ringer saline and a volume of 1 µl was injected into newly emerged adult females. Five days after injection fat body was removed (as described in section 2.2), total RNA was extracted from the tissue (see section 2.4), treated with DNase (section 2.5) and subjected to q-RT-PCR to quantify *vitellogenin* transcript levels (section 2.10). The phenotype of the treated insects was also analysed. The ovaries were removed and ovariole length measured using standard microscopy techniques.

3.3.2 M. sexta *RNAi*

Double-stranded RNA for injection into *Manduca sexta* was prepared as described in Section 2.11 and injected into larvae as described in section 2.2. In the majority of experiments the target was an immune-related gene and the larvae were injected with *E. coli* cells (approximately $5x10^5$ colony forming units resuspended in PBS) six hours after injection with dsRNA. An outline of the experimental scheme to target immune genes is shown in Figure 3.2a. The details of all the RNAi experiments conducted in *M. sexta* are presented in Table 3.1.

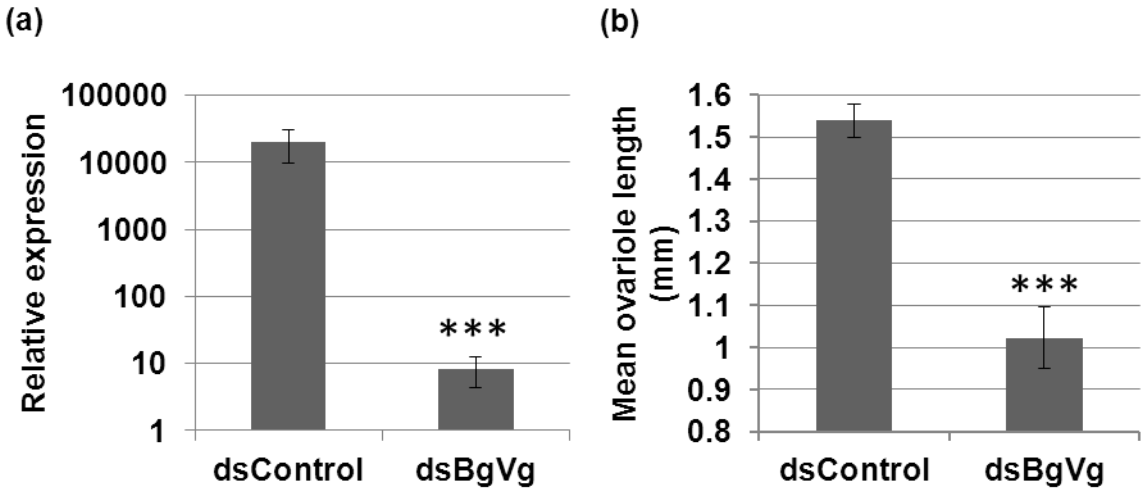


Figure 3.1: Silencing of *Blattella germanica vitellogenin (BgVg)* gene expression by *in vivo* RNAi. Newly emerged adult females were injected with 1 µg dsBgVg or dsControl and were analysed 5 days later. **(a)** q-RT-PCR of RNA extracted from fat bodies showing expression of vitellogenin (n=3). **(b)** Ovariole length (n=4). The following terminology was used for the p-values obtained from the t-tests performed: 0.05 (*), 0.01 (**), and 0.001 (***).

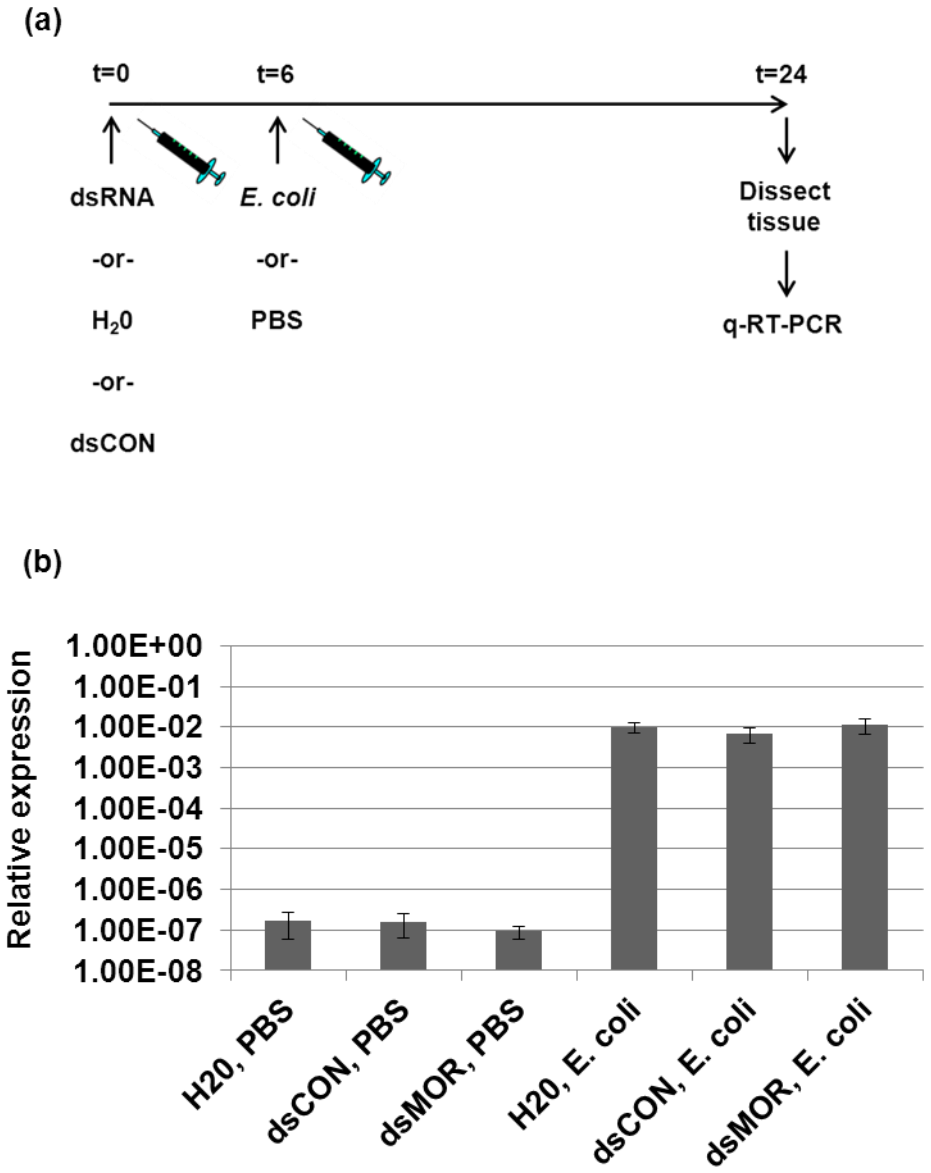


Figure 3.2: Attempt to silence *Manduca sexta moricin (MOR)* gene expression by *in vivo* RNAi. Newly emerged fifth instar larvae were injected with water (H20), 100ng of a double-stranded RNA control (dsCON) or 100ng dsRNA for moricin (dsMOR). Six hours post injection the insects were injected with either PBS or PBS containing approximately $5x10^5$ DH5α *E. coli* cells. Sixteen hours after injection with PBS or *E. coli* fat body tissue was collected from the insects and subjected to qPCR analysis to determine *moricin* transcript levels relative to transcript levels of the internal control gene, *RPS-3* (n=3).

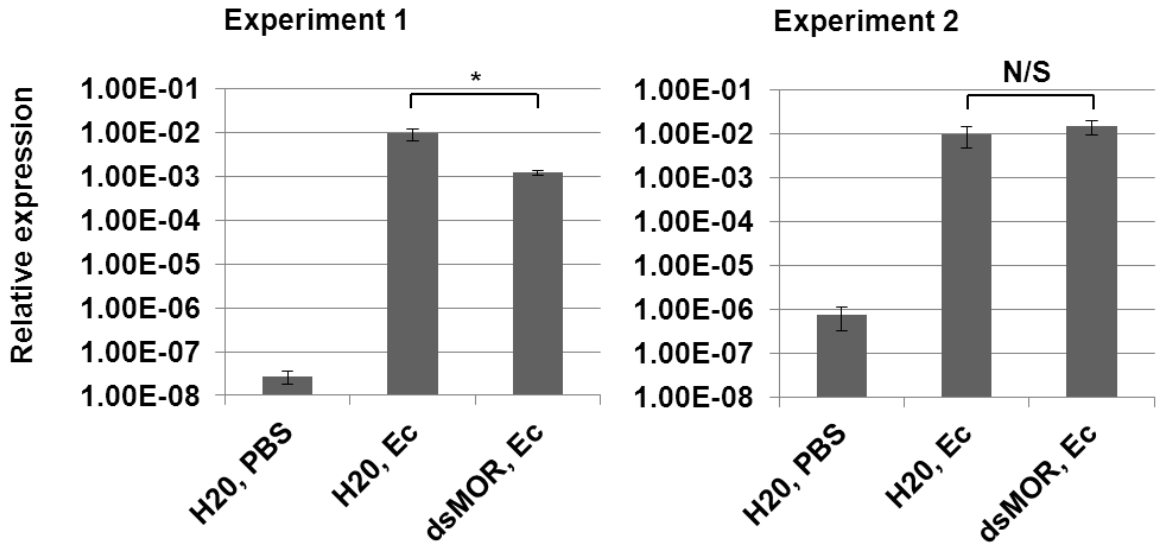


Figure 3.3: Varying efficacy of RNAi in *M. sexta*. Two RNAi experiments were conducted in order to silence the expression of *moricin*. Both experiments were conducted in the same manner. Newly emerged fifth instar larvae were injected with either water (H20) or 1µg of dsRNA for moricin (dsMOR). Six hours post injection the insects were injected with either PBS or PBS containing approximately $5x10^5$ DH5α *E. coli* cells. Sixteen hours after injection with PBS or *E. coli* hemocyte cells were collected from the insects and subjected to qPCR analysis to determine *moricin* transcript levels relative to transcript levels of the internal control gene, *RPS-3* (n=5). The following terminology was used for the p-values obtained from the t-tests performed to analyse differences in transcript levels of *E. coli* injected insects pre-injected with water or with dsRNA for *moricin*: >0.05 (N/S), 0.05 (*), 0.01 (**), and 0.001 (***).

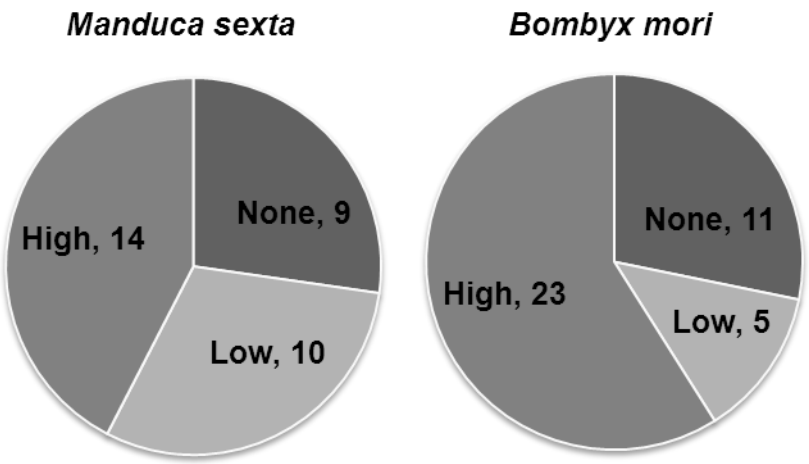


Figure 3.4: The outcome of RNAi experiments in *Manduca sexta* and *Bombyx mori*. The number of experiments reported during the study of Terenius *et al.* (2011) where the outcome was a high or low degree of silencing or no silencing (High, Low &None).

Table 3.1: Troubleshooting RNAi experiments in *M. sexta.* Details of the RNAi experiments conducted during this study.

Target	NCBI Accession	Induction	Dose	RNA delivery	dsRNA size (nts)	Developmental stage	Target tissue	Detection method	Result
Hemolin	U11879.1	*E. coli*	1ng, 10ng, 100ng & 1µg	Injection	613	5th instar larvae	Fat body	Semi-q RT-PCR & qPCR	No induction and no knockdown.
SPH3	AF413067.1	*E. coli*	100ng, 500ng & 1µg	Injection	657	5th instar larvae	Fat body	Semi-q RT-PCR & qPCR	No induction and no knockdown.
NOS	AF062749.1	*E. coli*	100ng	Injection	1045	5th instar larvae	Fat body	Semi-q RT-PCR	No induction and no knockdown.
IML-2	AF242202	*E. coli*	100ng	Injection	953	5th instar larvae	Fat body	qPCR	No induction and no knockdown.
Moricin	AY232301	*E. coli*	1ng, 10ng, 100ng & 1µg	Injection	216	5th instar larvae	Hemocytes & fat body	qPCR	Variable outcome.
vATPase subunits A, C and G	AJ249390.1, AJ249388.1 & X92805.1	None	200ng	Injection	822, 425&353	5th instar larvae	Midgut	Semi-q RT-PCR Growth rate of insects monitored	No knockdown. No reduction in growth rate.
vATPase subunits A, C and G		None	1µg & 10 µg	Feeding	822, 425&353	Neonates	Midgut	Growth rate of insects monitored	No reduction in growth rate.
vATPase subunit A		None	15µg	Injection	822	5th instar larvae	Midgut	Semi-q RT-PCR Growth rate of insects monitored.	No knockdown. No reduction in growth rate.
vATPase subunit C		None	1µg	Feeding	425	5th instar larvae	Midgut	Growth rate of insects monitored.	No reduction in growth rate.
Insecticyanin A, Insecticyanin B and mix of the two	X64714.1 & X64715.1	None	10µg	Injection	869&569	Late 4th, pharate 4th, 5th instar larvae	Cuticle	Observation of cuticle colour	No colour change

CHAPTER 4: THE PERSISTENCE OF DOUBLE-STRANDED RNA IN INSECT HEMOLYMPH DURING *IN VIVO* RNAI EXPERIMENTS

4.1 Introduction

RNA interference (RNAi) can be a useful tool for the functional characterisation of newly identified genes in insects (Mito *et al.*, 2011). Unfortunately, however, not all insect species are equally susceptible to RNAi (Terenius *et al.*, 2011). This thesis proposes several explanations for the variable RNAi response observed in different insect species; this chapter explores whether degradation of double-stranded RNA molecules (dsRNAs) during RNAi experiments could be responsible for the inadequate silencing of gene expression observed in some insects. In RNAi experiments in insects dsRNA is typically injected into the hemolymph. Since both dsRNA processing and the subsequent targeting and degradation of mRNA are cellular processes (Zamore *et al.*, 2000; Hammond *et al.*, 2000; Elbashir *et al.*, 2001*a*, 2001*b*; Nykänen *et al.*, 2001) in order for the RNAi experiment to be successful dsRNA must persist for sufficiently long in the hemolymph to allow the uptake and processing of dsRNA. Rapid degradation of dsRNA may result in insufficient uptake and subsequent processing of the dsRNA, and could, in some cases, be the limiting step in RNAi experiments. If persistence of dsRNA in insect hemolymph is a limiting factor in the success of RNAi experiments, and if the rate of degradation of dsRNA in the hemolymph varies significantly between different insect species, then this variation could explain the differing susceptibilities of insects to RNAi (i.e. insects in which dsRNA is rapidly degraded may be less susceptible to RNAi).

The energetic stability of dsRNA should be considered when speculating as to likely persistence rates in insect hemolymph. Single-stranded RNA molecules (ssRNAs) are well known to be unstable, breaking down in solution in conditions where DNA molecules are stable (Nicholson, 1996). This instability is a result of the action of 2'-hydroxyl groups, which behave as internal nucleophiles, attacking phosphodiester linkages and displacing the 5' oxygen of the neighbouring 3' nucleotide (Westheimer, 1968). Double-stranded RNA molecules form right-handed double helixes (with the A-helix motif) and are more stable than single-stranded RNA molecules (Nicholson, 1996) because their helical formation confers protection from 2'-hydroxyl attack on the 3'-5' internucleotide linkage (Usher, 1972). Thus, introduced dsRNA will persist longer than ssRNA, but nevertheless may break down from purely chemical causes and any experiment investigating dsRNA persistence must include appropriate controls to check this.

Precisely because of their energetic stability we can anticipate that introduced dsRNA molecules are likely to be targeted for degradation in insect hemolymph. Moreover, dsRNA is expressed by almost all viruses at some point during their replicative cycle and therefore represents an important viral pathogen-associated molecular pattern (PAMP) (DeWitte-Orr & Mossman, 2010). As a potential PAMP, dsRNA risks recognition by host pattern recognition receptors (PRRs), which are capable of inducing a barrage of specific responses (Hoffmann *et al.*, 1999). In vertebrates, Toll-like receptors (TLRs; in particular TLR3, 7, 8 and 9) detect viral nucleic acids in endosomal compartments (Kumar *et al.*, 2009*b*; Yoneyama & Fujita, 2010), whilst retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) recognise viral RNA in the cytoplasm (Yoneyama & Fujita, 2010). TLRs and RLRs activate signalling cascades to induce the gene expression of proinflammatory cytokines and type I interferons (IFNs). In insects less is known about the identity and function of dsRNA-sensing PRRs. One PRR candidate is the immunoglobulin family protein Hemolin, which is induced in *Antheraea pernyi* larvae by synthetic dsRNA and by nuclear polyhedrosis virus (*Ap*NPV) (Hirai *et al.*, 2004).

Endogenously expressed extracellular ribonuclease enzymes represent a potential source of anti-dsRNA activity in insect hemolymph. Several known classes of ribonucleases can degrade dsRNA. One example is the RNase III group of enzymes, which are active specifically against dsRNA molecules, possess endonucleolytic phosphodiesterase activity, and cleave dsRNA in a non-sequence-specific manner (Dunn, 1982; Nicholson, 1996). Whilst RNase III enzymes display activity specific to dsRNA molecules, other nuclease classes may also exhibit activity against dsRNA amongst their wider repertoire of enzymatic activities. DNA/RNA non-specific endonuclease enzymes, for instance, are able to degrade a wide range of nucleic acid molecules, including dsRNAs. A member of this class was recently isolated from digestive juices of the silkworm, *Bombyx mori* (Lepidoptera; Bombycidae), and named an "alkaline nuclease" because of its remarkably high isoelectric point (the pH at which the protein carries no net charge) (Arimatsu *et al.*, 2007*a*, 2007*b*).

In addition to the presence of endogenously expressed enzymes, insect hemolymph may also contain nucleases that have been produced by microorganisms. Whilst laboratory insect cultures may appear to be healthy and free of infecting microorganisms they may, in fact, be subject to numerous asymptomatic (or silent) infections. Viruses, for example, can cause latent infections, defined as infections during which infectious viral particles are not formed, and persistent infections, where virus particles are produced constantly at low levels (Dimmock & Primrose, 1987). Persistent and latent viral infections are suspected to be a very common phenomenon in insects naturally occurring in the field and in laboratory

colonies (Longworth & Cunningham, 1968; Chao *et al.*, 1985, 1986; Burand *et al.*, 1986; Podgwaite & Mazzone, 1986; Hughes *et al.*, 1993; Lee *et al.*, 1993). Moreover, viruses are known to produce RNases. For instances, Hussain *et al.* (2010) found that *Heliothis virescens* ascovirus (HvAV-3e) encodes an RNase III protein with dsRNA-specific endoribonuclease activity. Therefore, insect tissue may contain dsRNases produced by infecting viruses.

Along with organisms engaged in silent infections, symbiotic microorganisms are a potential source of nucleases. There is a wide range of known symbiotic associations of insects with bacteria, from the gut dwelling cellulolytic flagellates of termite species (Breznak & Brune, 1994) to the intracellular obligate symbionts of aphids (Akman Gündüz & Douglas, 2009). There is some evidence that infecting or symbiotic organisms may produce nuclease enzymes. Marcus & Yoshida (1990) revealed that the source of a dsRNase apparently produced by GMK Vero cells (a cell line derived from the kidney of the African green monkey) was in fact the infecting mollicute *Mycoplasma hyorhinis*. They then found that 11 further mycoplasma species (which belong to a class of bacteria distinguished by the absence of a cell wall) could also produce dsRNases. Since insects are also known to harbour infecting mollicutes (Hurst *et al.*, 1999; Jiggins *et al.*, 2000; Fukatsu *et al.*, 2001; Anbutsu & Fukatsu, 2003; Mateos *et al.*, 2006; Weinert *et al.*, 2007; Duron *et al.*, 2008; Watts *et al.*, 2009; Haselkorn *et al.*, 2009), mycoplasma species represent a realistic source of dsRNases in insects.

This study aims to determine the effect that the persistence of dsRNA may have on the success of *in vivo* RNAi experiments. To my knowledge the only study detailing the fate of dsRNA in insect hemolymph is that of Sakashita *et al.* (2009), which reported dsRNA-binding activity in *Bombyx mori* hemolymph, but did not report degradation of dsRNA or link this binding activity to RNAi susceptibility. This chapter evaluates the persistence of dsRNA in the hemolymph of the less RNAi-sensitive insect, *Manduca sexta*, as well as in hemolymph of the relatively RNAi-susceptible *Blattella germanica*. It will be shown that dsRNase activity is present in the hemolymph of *Manduca sexta.* As a result of this finding a number of experiments were conducted with the aim of identifying the source of the enzyme responsible, whether it be endogenously produced or the product of an infecting or symbiotic microorganism.

4.2 Results

4.2.1 dsRNA is degraded in vivo in Manduca sexta *hemolymph plasma*

In order to study the persistence of dsRNA in *M. sexta* hemolymph an assay based on the detection by quantitative reverse transcription PCR (q-RT-PCR) of injected dsRNA was developed. An absolute quantitation approach was employed and the assay calibrated using hemolymph plasma spiked with known quantities of dsRNA (Figure 4.1a). Plotting the qPCR output (Ct value, or cycle number) against log ng dsRNA allowed a linear trendline to be fitted to the calibration data, and the equation of the fitted line was subsequently used to calculate the quantity of dsRNA subjected to q-RT-PCR for a given qPCR signal.

When fitting the trendline to the calibration data it became apparent that the lowest dose used in the calibration assay did not fit the trend (grey diamond in Figure 4.1a). This dose of dsRNA gave a qPCR output (Ct value) the same as that generated from a non-dsRNA control (data not shown) and was therefore interpreted as falling below the detection limit of the assay and was excluded from the analysis. The detection limit itself was defined as the midpoint between the lowest dose of dsRNA (excluded from the trendline) and the lowest dose of dsRNA included in the trendline (Ct 22.79; Figure 4.1b).

The equation derived from the calibration assay was used to quantify dsRNA in *Manduca sexta* hemolymph plasma. Following injection of eGFP dsRNA into newly emerged fifth instar larvae hemolymph was collected a number of hours later, the cells removed by centrifugation and total RNA extracted from a known volume of the hemolymph plasma. An aliquot of total RNA was used for q-RT-PCR and the quantity of dsRNA per microlitre of hemolymph was calculated from the qPCR output (determined using the calibration equation) according to the appropriate dilution factors. Using this approach dsRNA was found to very rapidly disappear from the hemolymph plasma (Figure 4.1b; note the logarithmic scale). Indeed, linear regression analysis of the log transformed data (from the experiment denoted by the opened diamonds) found a significant negative relationship between dsRNA levels and the time post dsRNA injection ($t=-8.189$, $p<0.001$, $y=-0.66916x-0.39316$).

4.2.2 dsRNA is rapidly degraded in vitro in Manduca sexta *hemolymph plasma*

An *ex vivo* gel assay was developed in order to further investigate the persistence of dsRNA in insect hemolymph. In the assay dsRNA was incubated with dissected cell-free hemolymph and the integrity of the dsRNA subsequently analysed on a 1% agarose gel.

Incubation of dsRNA in cell-free hemolymph isolated from newly emerged fifth instar larvae of *Manduca sexta*, a relatively RNAi-refractory insect, resulted in weakening and smearing (corresponding to the appearance of smaller nucleic acid species) of the dsRNA band on the gel, which can be interpreted as degradation (Figure 4.2a). Smearing and weakening of the dsRNA band began to occur after incubation in hemolymph for just one hour; after three hours the dsRNA band had almost entirely disappeared, indicating very rapid degradation of dsRNA. The outcome of an experimental control, where dsRNA was incubated in water (Figure 4.2a: H_20, t=3), was that the dsRNA retained its integrity, allowing us to conclude that dsRNA is an energetically stable molecule and is not liable to spontaneously degradation. This control also eliminated the possibility that the agent responsible for the dsRNA degradation originated from a contaminant, rather than the insect hemolymph plasma itself.

In the experiment presented in Figure 4.1b dsRNA with the eGFP (non-*M. sexta*) sequence was used. Similar experiments were conducted using a number of different dsRNA molecules (bearing the *moricin*, *hemolin*, *SPH-3*, *NOS* and *vATPase* subunit A, C & G sequences, see Table 2.2 for details) and the outcome was always the same; rapid fading and smearing of the dsRNA band. Similarly, the outcome was the same regardless of the age of the insect. When experiments were conducted using hemolymph from larvae in the late fourth instar, from pharate fourth instar larvae, from larvae on days one to four of the fifth (and final) instar and from wandering larvae fading and smearing of the dsRNA band occurred (data not shown).

4.2.3 dsRNA is not rapidly degraded in Blattella germanica *hemolymph plasma*

Interestingly, when the *ex vivo* gel assay was conducted using hemolymph plasma from the relatively RNAi-susceptible species, *Blattella germanica*, little dsRNA degradation was observed (Figure 4.2b). In contrast to the smearing and weakening of the band of dsRNA observed following incubation in *M. sexta* hemolymph plasma, a discrete band of dsRNA was present following incubation in hemolymph from *B. germanica* adult females, even after incubation for 24 hours. From these observations it can be tentatively concluded that, whilst *M. sexta* hemolymph contains some extracellular agent with dsRNA-degrading capabilities, a similar compound is not present or active in *Blattella germanica* hemolymph.

4.2.4 An enzyme is implied in the degradation of dsRNA in Manduca sexta *hemolymph*

A number of experiments were conducted using the *ex vivo* gel assay in order to further elucidate the nature of the agent responsible for the degradation of dsRNA in *Manduca sexta* hemolymph plasma. These experiments revealed that the ability of *M. sexta* hemolymph plasma to degrade dsRNA was inhibited by pre-heating the hemolymph to 100ºC for 10 minutes (Figure 4.3a), suggesting that a heat-labile enzyme is responsible for the degradation. Degradation of dsRNA was also inhibited by addition to the hemolymph of the metal chelating agent EDTA (Figure 4.3b). The effect of EDTA was dose-dependent: 20 mM and 10mM EDTA completely inhibited dsRNA degradation (Figure 4.3b) whereas the addition of 5mM EDTA did not entirely inhibit degradation (data not shown). These results provide evidence that a metal-dependent enzyme is responsible for degradation of dsRNA in the hemolymph. Further experiments found that when hemolymph plasma was passed through 5 and 10 kDa filters the fractions retained in the filters retained the dsRNA-degrading activity, whilst the flow-through had no degrading activity (data not shown).

4.2.5 M. sexta encodes two "DNA/RNA non-specific endonucleases"

It would be of great interest to identify the enzyme responsible for the degradation of dsRNA in *M. sexta* hemolymph. One potentially relevant nuclease class is the "DNA/RNA non-specific endonuclease" family, a member of which was recently isolated from the digestive juices of the silkworm (*Bombyx mori;* Bombycoidea; Lepidoptera), and termed an "alkaline nuclease" because of its remarkably high isoelectric point (the pH at which the protein carries no net charge) of 9.48. In order to determine whether *M. sexta* encodes a similar "alkaline nuclease" gene a collection of EST libraries (found at https://insectacentral.org/) was interrogated using the *B. mori* sequence as a query in a tBLASTn search. This search revealed the presence of two sequences with similarity to the *B. mori* endonuclease, hereafter referred to as *Manduca sexta* alkaline nuclease 1 (MsAN1) and *Manduca sexta* alkaline nuclease 2 (MsAN2), in a larval midgut EST library. 1,547 bps of MsAN1 sequence and 1,543 bps of MsAN2 sequence were identified.

Translation of the *M. sexta* alkaline nuclease-like nucleotide sequences using a translation tool (found at http://web.expasy.org/translate/) revealed that they are both predicted to encode contain open reading frames (ORFs) encoding 447 amino acid residues. Analysis of their predicted domains revealed that they are both likely to encode a single DNA/RNA non-specific endonuclease domain and that they are identical in their domain architecture to the *Bombyx mori, Spodoptera littoralis* and *Spodoptera frugiperda* "alkaline nuclease" protein

sequences (Figure 4.4; domains were defined using the tool found at http://expasy.org/tools/scanprosite/). In addition the *M. sexta* proteins, like those encoded by *B. mori*, *S. littoralis* and *S. frugiptera,* contain a signal peptide. The *Manduca sexta* deduced sequences show high amino acid identity with the other lepidopteran sequences; MsAN1 has 76%, 72% & 71% identity with the *Bombyx mori, Spodoptera littoralis* and *Spodoptera frugiperda* sequences and MsAN2 66%, 68% & 68% identity. The high degree of amino acid identity of the *M. sexta* deduced amino acid sequences with other lepidopteran DNA/RNA non-specific endonucleases is evident when these sequences are aligned using ClustalW (Figure 4.5; http://www.ebi.ac.uk/Tools/msa/clustalw2/).

A phylogenetic tree constructed by the neighbour-joining method using ClustalW software revealed that the *M. sexta* alkaline nuclease proteins cluster, as one would expect, with the other lepidopteran DNA/RNA non-specific endonuclease proteins (Figure 4.6). The MsAN1 predicted protein appears to be most closely related to the *Bombyx mori* protein (and not the proteins from *Spodoptera littoralis* and *Spodoptera frugiperda*), which is not unexpected given the relatively short phylogenetic distance between *M. sexta* and *B. mori* (Regier *et al.*, 2009). The MsAN2 predicted protein, however, does not cluster with the *B. mori* protein, but is instead placed on a branch of its own (Figure 4.6), reflecting its lower amino acid identity with the *Bombyx* DNA/RNA non-specific endonuclease (66% identity compared to 76% for MsAN1). Interestingly, the MsAN2 predicted protein still shares 75% amino acid identity with the *Bombyx* protein when only the DNA/RNA non-specific endonuclease domain is analysed, with much of the sequence variation occurring in the region outside this functional domain (Figure 4.7; MsAN2 has only 54% identity with the *Bombyx* protein in the regions not encoding the DNA/RNA non-specific endonuclease domain). In addition to having lower amino acid identity with the *Bombyx mori* alkaline nuclease protein (compared with MsAN1), the MsAN2 predicted protein further differs from the *Bombyx* protein in its isoelectric point (as predicted by the ProtParam tool found at http://expasy.org/tools/protparam.html). MsAN2 has an isoelectric point (pI) of 7.97 compared to 9.48 for the *Bombyx* protein and 9.32 for the MsAN1 predicted protein (Figure 4.6).

4.2.6 M. sexta *alkaline nuclease 1&2 are expressed at low levels in larval tissue*

Following the identification of two sequences in the *M. sexta* transcriptome with a high degree of similarity to DNA/RNA non-specific endonucleases from other lepidopteran species, it was interesting to ask whether these gene products could be responsible for the degradation of dsRNA previously observed in *M. sexta* larval hemolymph. In order to be

active in *M. sexta* larvae the two genes must be expressed in larval tissues. We know that the two genes certainly are expressed to some extent in larval midgut tissue, since they were identified in a larval midgut EST library. However, q-RT-PCR experiments revealed that expression levels of the transcripts in larval tissue (fat body, hemocytes and midgut) are extremely low (Figure 4.8a). Transcripts of *M. sexta* alkaline nuclease 1 are present at resting levels approximately 1,000,000 times lower than the internal control gene, RPS-3, in fat body, 100,000 times lower than the internal control gene in hemocytes and 10,000 times lower than the internal control gene in midgut tissue. Transcripts of *M. sexta* alkaline nuclease 2 are present in similar levels to those of *M. sexta* alkaline nuclease 1 in the midgut, but were undetectable using our q-RT-PCR methodology in the fat body and hemocytes (and must therefore be expressed at levels more than 1,000,000 times lower than the internal control gene).

It might, nevertheless, be the case that these two genes are only expressed under conditions of exposure to dsRNA. Comparison of transcript levels in insects injected with a water control or with dsRNA revealed, however, that MsAN1 transcript levels were not significantly elevated compared to the control in insects injected with dsRNA in fat body, hemocytes and midgut at 6 hours post injection with dsRNA (Figure 4.8b; t-tests: fat body $t=0.295$, $p=0.778$, hemocytes $t=-1.021$, $p=0.347$, midgut $t=-1.179$, $p=0.283$). There was also no significant increase in the expression of MsAN2 in the midgut of dsRNA-injected insects (Figure 4.8b; $t=-0.938$, $p=0.4013$). The expression of MsAN1 and MsAN2 were subsequently found to be constant (and not elevated) at 18 hours post dsRNA injection (data not shown). Thus, quantitative PCR reveals that the alkaline nucleases MsAN1 and MsAN2 are expressed at extremely low levels in *M. sexta* larval tissue and that their expression is unresponsive to dsRNA injection and that they are, therefore, unlikely to be involved in dsRNA degradation in larvae.

4.2.7 Detection of viral particles in Manduca sexta *tissue*

Since latent or persistent viral infections may be a source of nuclease enzymes, it seemed sensible to ask whether the agent responsible for dsRNA degradation in *M. sexta* hemolymph may have originated from an infecting virus. The observation that resting levels of hemolin, a gene implicated in viral defence (Hirai *et al.*, 2004), were relatively high (C. Fleury, personal communication, 2009) gave an indication that the laboratory colony was silently infected by a virus. Negative staining of fifth instar *M. sexta* larval tissue with uranyl oxylate followed by transmission electron microscopy (TEM) revealed the presence of virus-like particles in fat body and hemocytes (Figure 4.9). The size and the regular shape of the

particles indicate that they are likely to be virus particles. In fact, their form is remarkably similar to the Flock House Virus particles imaged by Walukiewicz *et al.* (2006) (Figure 4.9b).

4.2.8 No evidence for mycoplasma infection in the Manduca sexta *colony*

Just as infecting viruses may be a potential source of nucleases in insect hemolymph, mollicute species could equally be responsible for the presence of dsRNase molecules in *M. sexta* hemolymph plasma. A PCR-based detection approach utilising the universal (and slightly degenerate) mycoplasma primers described by Sung *et al.* (2006) was employed. These primers had been carefully designed to anneal to regions conserved across the mollicute taxa, and their use therefore results in successful amplification from a mycoplasma template (and no amplification from a non-mycoplasma template). When genomic DNA from *Drosophila melanogaster* "Red" adult females (known to harbour infecting mollicutes; G. Hurst, personal communication, 2009) was used as a template in the PCR a product of the appropriate size was obtained (Figure 4.10, L1). However, no appropriate bands were obtained when genomic DNA from first instar *M. sexta* larvae (Figure 4.10, L2), third instar *M. sexta* larvae (Figure 4.10, L3) and fat body from fifth instar larvae (Figure 4.10, L4) was used as the PCR template. The integrity of the *M. sexta* genomic DNA was confirmed by the successful amplification of the internal control gene, RPS-3 (Figure 4.10, L6-8).

4.3 Discussion

This chapter reports data concerning the persistence of dsRNA in insect hemolymph and reflects on the effect that the degradation of dsRNA may have on the outcome of RNAi experiments. It was hypothesised that rapid degradation of dsRNA could be responsible for the observed deficient response of some insect species to RNAi (Terenius *et al.*, 2011) and that variability in the persistence of dsRNA in insect hemolymph may explain the variation in the efficacy of RNAi in insects. In order to address these hypotheses a number of techniques to detect dsRNA in insect hemolymph were developed and used to determine the persistence of dsRNA in two insect species.

Several experiments conducted during this study indicate that synthetic dsRNA molecules are rapidly degraded in *Manduca sexta* hemolymph. A q-RT-PCR-based dsRNA detection assay found that injected dsRNA rapidly disappears from hemolymph plasma *in vivo* (Figure 4.1b). These results led to the hypothesis that dsRNA is degraded by some agent in *M. sexta*

hemolymph, although they do not in themselves confirm degradation, since the observed disappearance of the dsRNA signal from the hemolymph may have resulted from the uptake of dsRNA into cells.

To further study the persistence of dsRNA in *Manduca sexta* hemolymph, an *ex vivo* degradation assay, where dsRNA was incubated in hemolymph plasma, was developed. This assay had the advantage of being able to detect dsRNA in a cell-free system, thereby eliminating the possibility that any disappearance of dsRNA could be explained by uptake into cells. When dsRNA was incubated with *M. sexta* hemolymph plasma and its integrity subsequently analysed by gel electrophoresis it became clear that the dsRNA had been degraded by a hemolymph component (Figure 4.2a). Further experiments using the *ex vivo* degradation assay revealed that the degradation of dsRNA by hemolymph plasma is inhibited by previous boiling of the plasma and also by inclusion in the reaction mix of 10mM EDTA (Figure 4.3), indicating that a heat-labile proteinaceous molecule with a requirement for cations is responsible for the degradation. These results point to the presence of an unknown and probably constitutively expressed extracellular dsRNA-degrading enzyme in the hemolymph of *M. sexta* larvae.

Following the observation that dsRNA is rapidly degraded in *M. sexta* hemolymph the question arose of whether the degradation of dsRNA is likely to be responsible for the observed inefficiency of RNAi in this insect. It has already been well documented in the literature that the action of endogenously expressed nucleases can be important in determining the outcome of RNAi experiments. In the nematode worm, *C. elegans*, it was observed that a nuclease encoded by the *eri-1* (*enhanced RNAi-1*) gene is expressed at high levels in the gonad and neurons of the worm, tissues which are unusually refractory to RNAi (all other tissues in *C. elegans* appear to be susceptible to RNAi) (Kennedy *et al.*, 2004). Moreover, *eri-1* mutant worms show an enhanced RNAi effect and accumulate more siRNAs than wild type animals (Kennedy *et al.*, 2004). The role played by nucleases in RNAi experiments is not limited to nematode worms. An *eri-1* homologue was discovered in mice (*meri-1*: mouse enhanced RNAi-1) and RNAi targeted to this gene resulted in enhanced sensitivity and efficiency of RNAi (Hong *et al.*, 2005). There are no examples known to me, however, of insect nucleases which interfere with RNAi, although dsRNA-binding activity has been reported in the hemolymph of *Bombyx mori* (Sakashita *et al.*, 2009).

To address the question of whether degradation of dsRNA is likely to be responsible for the observed inefficiency of RNAi in *Manduca sexta* a comparison was made between degradation of dsRNA in the hemolymph of the RNAi-resistance species *M. sexta* and

degradation in an RNAi-susceptible insect species. The RNAi-sensitive species the German cockroach, *Blattella germanica* (Blattodea; Blattellidae), was chosen for study. After confirmation of the susceptibility of this species to RNAi (by conducting a successful RNAi experiment to knock down the vitellogenin gene in adult female cockroaches, see Chapter 3 Figure 3.1) the survival of dsRNA in *B. germanica* hemolymph was investigated using the *ex vivo* gel assay. Interestingly, there did not appear to be any degradation of dsRNA following incubation in the hemolymph of this RNAi-susceptible species (Figure 4.2b).

The finding that rapid degradation of dsRNA occurs in the hemolymph of the RNAi-insensitive *M. sexta,* but not in the hemolymph of the RNAi-sensitive *B. germanica* indicates that degradation of dsRNA (or at least the rate of degradation of dsRNA) may be a key factor in determining the success of RNAi experiments. However, caution is required when drawing conclusions from these data, since the results presented here only provide evidence of the degree of degradation in one RNAi-insensitive and one RNAi-sensitive species. Furthermore, these two species are phylogenetically distant. A more interesting and relevant comparison could be made of degradation of dsRNA in closely related insect species with differing susceptibilities to RNAi.

Having observed this difference in degradation between *Manduca sexta* and *Blattella germanica* it is interesting to speculate as to potential evolutionary explanations for this difference. Since dsRNA can be recognised as a viral molecular pattern (DeWitte-Orr & Mossman, 2010) rapid degradation of dsRNA in hemolymph could represent an evolutionary response to a heavy viral load. This explanation is plausible, since lepidopteran species like *Manduca sexta* appear to be particularly prone to viral infections (Terenius, 2008). For instance, Martignoni & Iwai (1986) found that, whilst lepidopteran insects only make up 20% of all described holometabolic species, 70% of holometabolic species with recorded viral disease in their study belonged to the Lepidoptera. Furthermore, there are many examples of Lepidoptera-specific viruses, including viruses from the genera *Granulovirus* (Baculoviridae) (Granados & Federici, 1986; Bilimoria, 1991; Miller, 1997). In contrast, few viruses are known to be associated with cockroaches (insects of the Order Blattodea). Evans & Shapiro (1997) reported that the densonucleosis virus (DNV; Parvoviridae, Densovirus) is the only virus infecting cockroaches. Although an entomopoxvirus has subsequently been isolated from *Blattella germanica* (Radek & Fabel, 2000), there is very little evidence of infection by viruses in cockroaches: conducting a Web of Science search for "virus blattodea" returns no hits and searching for "virus blattella" returns just 9 hits.

Returning now to the issue of degradation of dsRNA in *M. sexta* hemolymph, and the supposition that rapid degradation of dsRNA is responsible for the inefficacy of RNAi in this insect, it may also follow that other insect species that are refractory to RNAi are experiencing rapid degradation of dsRNA in the hemolymph, and that degradation is the underlying cause of the inefficiency of RNAi. If this is so, then what can be done to improve the efficiency of RNAi experiments in these insects? As was touched upon previously with reference to the *meri-1* gene in mice (Hong *et al.*, 2005), nucleases which interfere with RNAi can themselves be targeted and silenced by RNAi resulting in enhanced sensitivity and efficiency of RNAi. In order to apply this approach one must first identify the nuclease responsible for degradation, so that dsRNA with the cognate sequence can be designed, synthesised and deployed.

In order to identify the nuclease responsible for degradation of dsRNA in *M. sexta* examples of endogenously expressed dsRNases were sought. Although no such enzymes had previously been identified in *M. sexta*, a ribonuclease active against dsRNA was recently isolated from *Bombyx mori* digestive juices (Arimatsu *et al.*, 2007*a*, 2007*b*). This enzyme belonged to the "DNA/RNA non-specific endonuclease" enzyme class and was named an "alkaline nuclease" because of its high isoelectric point. Analysis of the *M. sexta* transcriptome revealed that this insect, in fact, encodes two homologues of this *Bombyx* gene, which are referred to as *Manduca sexta* alkaline nuclease 1 (MsAN1) and *Manduca sexta* alkaline nuclease 2 (MsAN2).

Interestingly, MsAN1 is very similar to the *Bombyx* enzyme in its amino acid identity and predicted isoelectric point, whilst the MsAN2 protein shares rather less amino acid identity with the *Bombyx* protein and has a rather lower predicted isoelectric point (Figures 4.5 & 4.6). These observations lead to generation of the hypothesis that, following a gene duplication event in *Manduca sexta*, the MsAN2 gene product has diverged in function. The MsAN2 protein does show a high degree of amino acid conservation with the *Bombyx* protein in its functional "DNA/RNA non-specific endonuclease" domain (Figure 4.7), and is therefore likely to have retained its function as an endonuclease. The pH at which the proteins are predicted to operate may provide a clue as to any potential divergence in function of the MsAN2 protein. Whilst the *Bombyx* protein is active under highly alkaline conditions (Arimatsu *et al.*, 2007*a*) and the MsAN1 protein is predicted to be active under highly alkaline conditions (with a predicted pI of 9.32), the MsAN2 protein is predicted to be active under rather less alkaline conditions (with a predicted pI of 7.97). The activity of the *Bombyx* nuclease in alkaline conditions may be explained by its location in the midgut lumen (Arimatsu *et al.*, 2007*a*), since lepidopteran larval guts have an extremely high luminal pH of between 9 and 12 (Waterhouse, 1949; Dow, 1984). This leads to the

prediction that the MsAN1 protein is present in the midgut lumen, whilst the MsAN2 protein is functioning elsewhere, perhaps in the hemolymph, which generally has a pH of around 6.7 (Dow, 1984).

The identification of two "DNA/RNA non-specific endonuclease" type nucleases in the *M. sexta* transcriptome led to the question; is it likely that either one is responsible for the degradation of dsRNA in the larval hemolymph? The prediction that the MsAN2 gene product functions at a lower pH than the MsAN1 gene product leads to the hypothesis that this enzyme is more likely to be active in insect hemolymph. Expression analyses revealed, however, that both *M. sexta* genes are expressed at very low levels in the three larval tissues tested (fat body, hemocytes and midgut: Figure 4.8a) and that both are expressed at higher levels in midgut tissue compared to hemocytes and fat body (MsAN2 mRNA was actually undetectable in hemocytes and fat body). There is, therefore, no evidence for the expression of MsAN2 in those tissues that may release protein into the hemolymph (the hemocytes and fat body). This finding, together with the observation of extremely low expression levels of both nuclease genes, leads to the conclusion that it is unlikely that the proteins encoded by these newly identified genes are responsible for the degradation of dsRNA observed in *M. sexta* hemolymph. However, it is possible that even very low levels of nuclease could be sufficient to give rise to the degrading phenotype if the specific activity was high enough.

If the expression levels of the newly identified *M. sexta* nucleases were found to be induced in response to dsRNA this would potentially provide support for the hypothesis that they are active against dsRNA in larval hemolymph. Transcriptional regulation cannot be responsible for the nuclease activity observed in *M. sexta* hemolymph, because degradation of dsRNA occurred in cell-free hemolymph plasma. However, responsiveness to dsRNA may implicate the *M. sexta* nucleases in the degradation of dsRNA, since DNA/RNA non-specific endonuclease expression levels have been reported to respond to dsRNA. Bao *et al.* (2008) found that the *Bombyx mori* alkaline nuclease gene isolated and characterised by Arimatsu *et al.* (2007*a*, 2007*b*) was upregulated in *B. mori* midgut tissue following infection with a densonucleosis virus (BmDNV-Z), representing a dsRNA challenge. However, this study found that dsRNA injection did not result in significantly elevated MsAN1 and MsAN2 transcript levels in *M. sexta* (Figure 4.8b), suggesting that these nucleases may not be involved in degradation of dsRNA in *M. sexta* larvae.

As well as the action of endogenously expressed nucleases, infecting agents may also represent a potential source of dsRNase activity. For example, mycoplasma species are known to produce dsRNase molecules (Marcus & Yoshida, 1990) and could be responsible

for the degradation of dsRNA in *Manduca sexta* hemolymph. However, PCR-based detected techniques revealed the absence of mycoplasma DNA in *M. sexta* tissue, indicating that the insects are unlikely to be infected by mycoplasma species.

In contrast, virus-like particles were detected in *M. sexta* fat body and hemocyte tissue using transmission electron microscopy. Taken together with the observation that the colony used for these experiments was observed to have remarkably high resting transcript levels of hemolin (C. Fleury; personal communication, 2009), which is known to be responsive to dsRNA and viral infection in the lepidopteran *Antheraea pernyi* (Hirai *et al.*, 2004), this leads to the conclusion that the *M. sexta* colony is subjected to at least one asymptomatic viral infection.

As demonstrated by Hussain *et al.* (2010) viruses are able to produce proteins that can degrade dsRNA and, therefore, virus-derived dsRNase enzymes could be responsible for the observed degradation of dsRNA in *M. sexta* larvae. In order to confirm that an infecting virus was directly interfering with RNAi one approach would be to attempt to "cure" the *M. sexta* colony of its infection (perhaps by the administration of an anti-viral agent) and observe whether the treated insects showed an improved ability to perform RNAi. A positive result in an experiment such as this would provide a link between an infecting virus and insensitivity to RNAi. It must be considered, however, that an infecting virus may affect the outcome of RNAi experiments in a number of ways, not just by the production of dsRNA-specific nucleases. The flock house virus (FHV; *Nodaviridae*), for instance, which has been reported to latently infect *Drosophila melanogaster* laboratory colonies, encodes a suppressor of RNAi (Li *et al.*, 2002), which is thought to act by preventing the processing of long dsRNA in siRNAs by Dicer (Sullivan & Ganem, 2005; Lu *et al.*, 2005).

In summary, *M. sexta* larval cell-free hemolymph was found to contain significant nuclease activity against dsRNA. In contrast, the hemolymph of *Blattella germanica*, which, unlike *M. sexta*, is highly sensitive to RNAi, did not contain any observable dsRNA-specific nuclease activity. Several potential sources the nuclease activity were considered, including the endogenously expressed alkaline nuclease 1 and alkaline nuclease 2 proteins, as well as agents produced by infecting mollicutes and viruses. Unfortunately, in the end it was not possible to identify the nuclease responsible for the degradation of dsRNA.

4.4 Methods

4.4.1 In vivo *dsRNA detection assay*

Quantitative reverse transcription PCR (q-RT-PCR) was used to detect injected eGFP dsRNA in insect hemolymph. Initial experiments confirmed that q-RT-PCR, which is typically used for the detection of single-stranded messenger RNA (mRNA), could also be used to detect double-stranded RNA. The step in the q-RT-PCR protocol directly preceding the reverse transcription reaction involves heating RNA to 75°C for 10 minutes to denature RNA secondary structure. This heating step separates double-stranded RNA into single-stranded molecules, allowing reverse transcription of the RNA (separation of RNA strands was confirmed using gel electrophoresis; data not shown).

In order to quantify dsRNA levels in hemolymph, the detection of eGFP dsRNA by q-RT-PCR was calibrated using dissected hemolymph plasma spiked with a range of known doses of dsRNA for eGFP. It was necessary to add 10 µg Poly cytidylic-inosinic acid potassium salt (Poly(I:C); supplied by Sigma) to the hemolymph plasma to act as a carrier in the extraction of RNA because plasma itself does not contain sufficient quantities of RNA for extraction to be successful. q-RT-PCR was carried out as previously described (Chapter 2, section 2.10) using primers eGFP_qPCR_F: 5'-CCG ACC ACT ACC AGC AGA AC-3' and eGFP_qPCR_R: 5'-TTG GGG TCT TTG CTC AGG-3'. The calibration experiment gave rise to a formula capable of quantitating dsGFP levels in plasma for a given qPCR signal (Figure 4.1a).

The formula derived from the calibration experiment was used to quantify eGFP dsRNA in hemolymph plasma. Newly emerged fifth instar *M. sexta* larvae were injected with 4 µg dsRNA for eGFP and dissected for hemolymph plasma after incubation for a number of hours at 25°C. 10 µg Poly(I:C) was once again added to the hemolymph plasma to act as a carrier during the extraction of RNA. Total RNA was extracted from the tissue, treated with DNase and an aliquot reverse transcribed, as described in section 2.10, except that the amount of RNA used in the reverse transcription reaction was adjusted for all samples to be equivalent to that extracted from 20 µl hemolymph plasma. q-RT-PCR was performed on a dilution of the resulting cDNA and Ct values obtained from the qPCR reactions were used to calculate the quantity of dsRNA in the tissue using the formula derived from the calibration experiment (and any dilution factors).

4.4.2 Ex vivo *dsRNA degradation assay (gel assay)*

A gel-based detection assay was developed in order to test the integrity of dsRNA following exposure to *M. sexta* or *B. germanica* hemolymph. 1 µl dsRNA solution (1 µg μl^{-1}*;* details for the preparation of dsRNA are found in section 2.11) was mixed with 3 µl insect cell free hemolymph in a 1.5 µl microcentrifuge tube and incubated at room temperature for a number of hours. Cell free hemolymph was prepared as described in section 2.2 except the hemolymph was bled into a microcentrifuge tube containing a few grains of phenylthiourea (PTU) to inhibit melanisation (Arakawa, 1995). In a control treatment dsRNA was incubated with DEPC-treated water (Ambion) for the same time as the longest experimental incubation. After incubation in hemolymph the dsRNA was recovered using an RNeasy Mini Kit (Qiagen): dsRNA was bound onto silica membranes in spin columns allowing contaminants to be washed away prior to the elution of the RNA. Bound dsRNA was eluted into 30 µl DEPC-treated water and a 10 µl aliquot mixed with 2 µl loading dye (New England Biolabs) and run on a 1% agarose EtBr gel (as described in section 2.7). Visualisation of the gel using a UV transilluminator allowed analysis of the integrity of dsRNA.

4.4.3 Identification of DNA/RNA non-specific endonucleases in Manduca sexta *transcriptome*

Several *M. sexta* expressed-sequence-tag (EST) and 454 sequencing libraries (found at insectacentral.org) were interrogated. A tBLASTn search, which searches translated nucleotide databases using a protein query, was performed in order to identify *M. sexta* sequences with similarity to the *Bombyx mori* DNA/RNA non-specific endonuclease (or alkaline nuclease) gene (BAF33251.1). Contigs with similarity to the *Bombyx* sequence were assembled and aligned, revealing two *M. sexta* alkaline nuclease-like transcripts with good coverage over the entire length of the sequences. Good coverage of the genes allowed the manual correction of any sequencing errors and the final assembly of the two genes.

4.4.4 Detection of viral particles in M. sexta *tissue*

In order to detect viral particles in *M. sexta* larval tissue, a negative staining transmission electron microscopy (TEM) protocol was developed. Fat body tissue and hemocytes were dissected from newly emerged fifth instar *M. sexta* larvae as previously described (section 2.2). Dissected tissue was homogenised in fixative solution {2.5% glutaraldehyde, 2%

paraformaldehyde and 2.5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer (pH 7.4)} by crushing with a pipette tip and incubated in fixative overnight at 4°C. Tissue samples were then rinsed to remove the fixative and washed three times with 0.1 M sodium cacodylate. The last wash solution was removed and replaced with 1% osmium tetroxide and1% potassium ferrocyanide and incubated for 1 hour at room temperature. The tissues were then washed three times in distilled water and stained by incubating with 2% aqueous uranyl oxylate for 1 hour in the dark. The samples were dehydrated progressively in acetone with two changes of 50%, 70%, 90% & 95% solutions on ice and four changes of 100% dry acetone at room temperature and infiltrated in Spurrs Epoxy Resin overnight at room temperature on a stirrer. On the following day the tissue samples were embedded in fresh Spurrs Epoxy Resin and polymerised in an oven at 70°C for seven hours. After polymerisation the resin blocks were sectioned at 60 nm on a Reichert OMU3 microtome using glass knives (TAAB) and collected onto copper palladium grids. Samples were analysed on a JEOL JEM1200EXII transmission electron microscope.

4.4.5 Detection of mycoplasma in M. sexta *tissue*

PCR amplification with carefully designed universal primers was used to detect mycoplasma species in *Manduca sexta* tissue. Genomic DNA was extracted from first and third instar *M. sexta* larvae, from fat body derived from fifth instar larvae and from *Drosophila melanogaster* "Red" adult females (which acted as a positive control since this line is known to harbour mycoplasma species). To conduct the extraction two *D. melanogaster* individuals, two *M. sexta* first instar larvae, one third instar larva and approximately 500 ng fat body tissue was homogenised with a pipette tip in 100 µl 1.25% aqueous ammonia (NH_4OH) in microcentrifuge "safe-lock" tubes (Eppendorf) (300 µl aqueous ammonia was used for the *M. sexta* third instar larva due to its relatively large mass). Following homogenisation the samples were heated at 100°C in a block heater for 20 minutes, after which they were removed from the heat. The tubes were opened and heated again at 100°C until approximately 25% of the liquid had evaporated. A DNeasy Blood and Tissue Kit (Qiagen) was thereafter used to purify the extracted DNA. The samples were applied to membranes in the spin columns provided and the "Purification of DNA from Animal Tissue" protocol was followed in order to obtain purified genomic DNA.

Following the quantification of the extracted genomic DNA using spectrophotometry 10 ng of DNA was added to thin walled PCR tubes (Sarstedt) containing PCR reagents (as described in section 2.6). The universal mycoplasma primers used were those described by

Sung *et al.* (2006). The forward primer (5'-ACA CCA TGG GAG (C/T)TG GTA AT-3') originated from a study by Harasawa *et al.* (1993) and the reverse primer (5'-CTT C(A/T)T CGA CTT (C/T)CA GAC CCA AGG CAT-3') from the work of Nakagawa *et al.* (1992). The reaction conditions used were heating at 95°C for 5 minutes for initial denaturation, followed by followed by 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute with a final extension of 72°C for 5 minutes. In order to confirm the quality and integrity of the *M. sexta* genomic DNA, control reactions using RPS-3 primers (Forward 5'-CTG GCT GAG GAT GGC TAC TC-3' & Reverse 5'-TTT CTC AGC GTA CAG CTC CA-3') were set up alongside mycoplasma reactions. Amplification products were analysed by gel electrophoresis as described in section 2.7, except that the concentration of the agarose gel was 1.5 %.

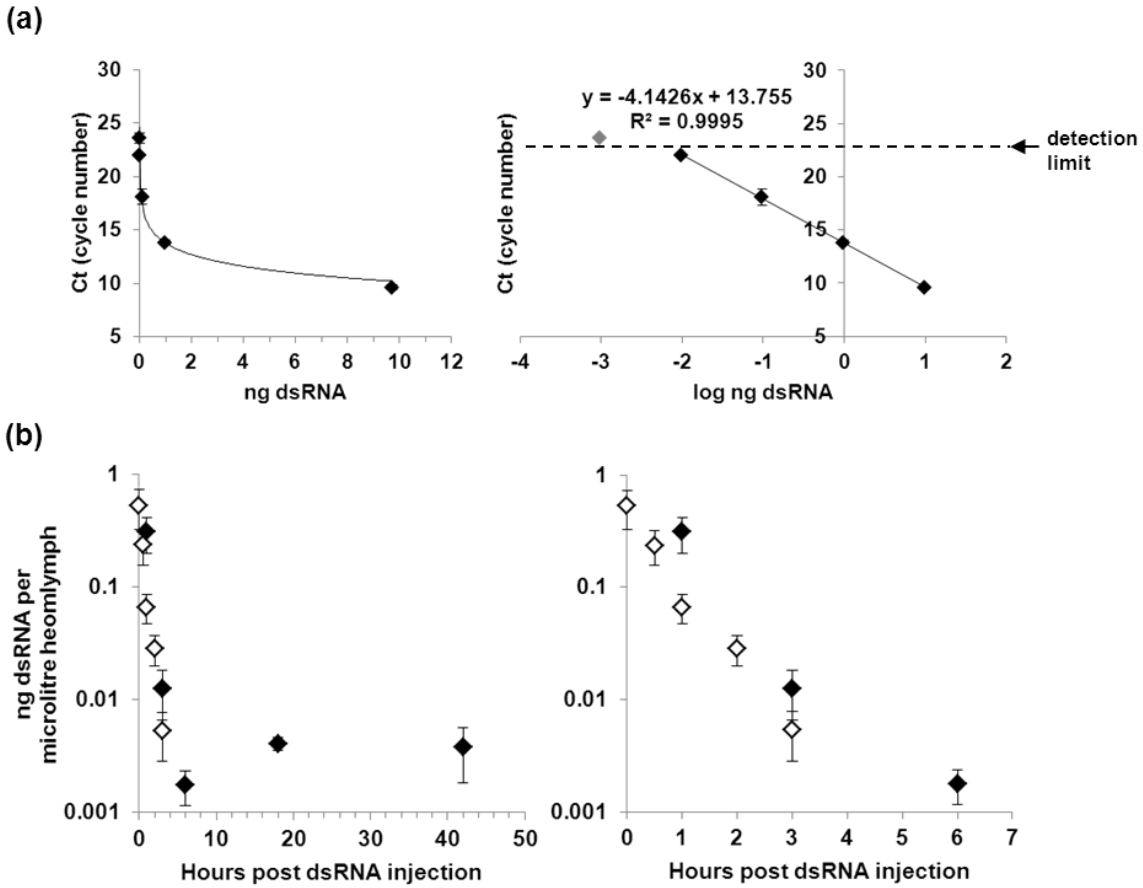


Figure 4.1: Persistence of dsRNA in *Manduca sexta* hemolymph. (a) The assay was calibrated by spiking dissected plasma with a range of doses of dsRNA for eGFP and by carrying out q-RT-PCR. The qPCR output (Ct, or cycle number) was plotted against the quantity of dsRNA (left) and the log transformed quantity of dsRNA (right). One data point (in grey) was excluded from the fitted trendline because it was interpreted as falling below the detection limit of the assay. **(b)** Two *in vivo* persistence assays were conducted, where 4µg of eGFP dsRNA was injected into newly emerged fifth instar larvae and the hemolymph dissected after incubation for a number of hours (n=4). Hemocytes were removed from the hemolymph by centrifugation and absolute dsGFP levels in the plasma quantified using the calibrated q-RT-PCR protocol. The first experiment (depicted with black diamonds) monitored dsRNA levels over 48 hours, whilst the second experiment (open diamonds) concentrated on shorter time points. The two scatter plots depict the same data but the plot on the right displays data for the first six hours only.

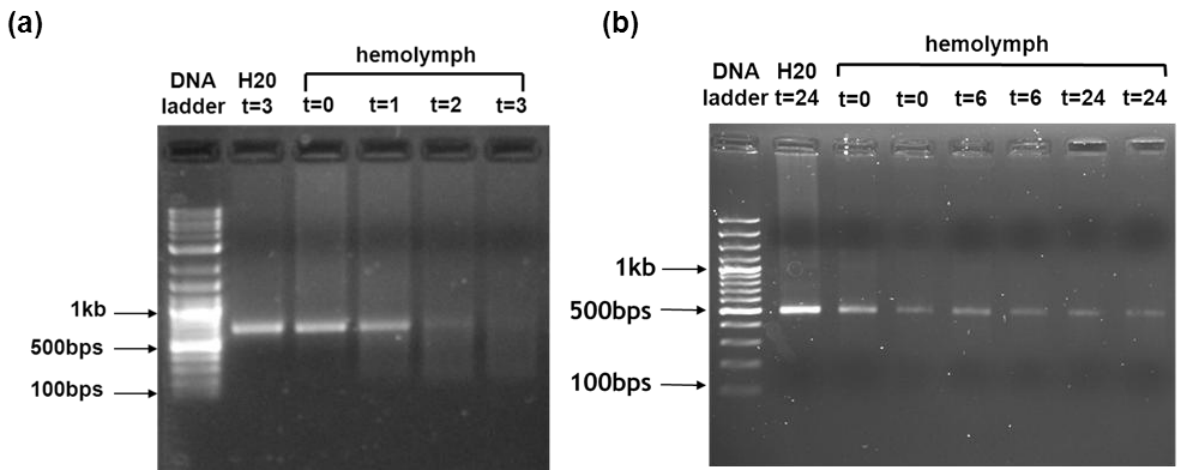


Figure 4.2: Degradation in (a) *Manduca sexta* and (b) *Blattella germanica*. 1 µg dsRNA for eGFP was incubated with DEPC-treated water (H_20) or insect cell-free hemolymph for t hours after which it was recovered using an RNeasy kit (Qiagen) and run on a 1% agarose EtBr gel. Hemolymph was prepared from *M. sexta* fifth instar larvae and *B. germanica* adult females.

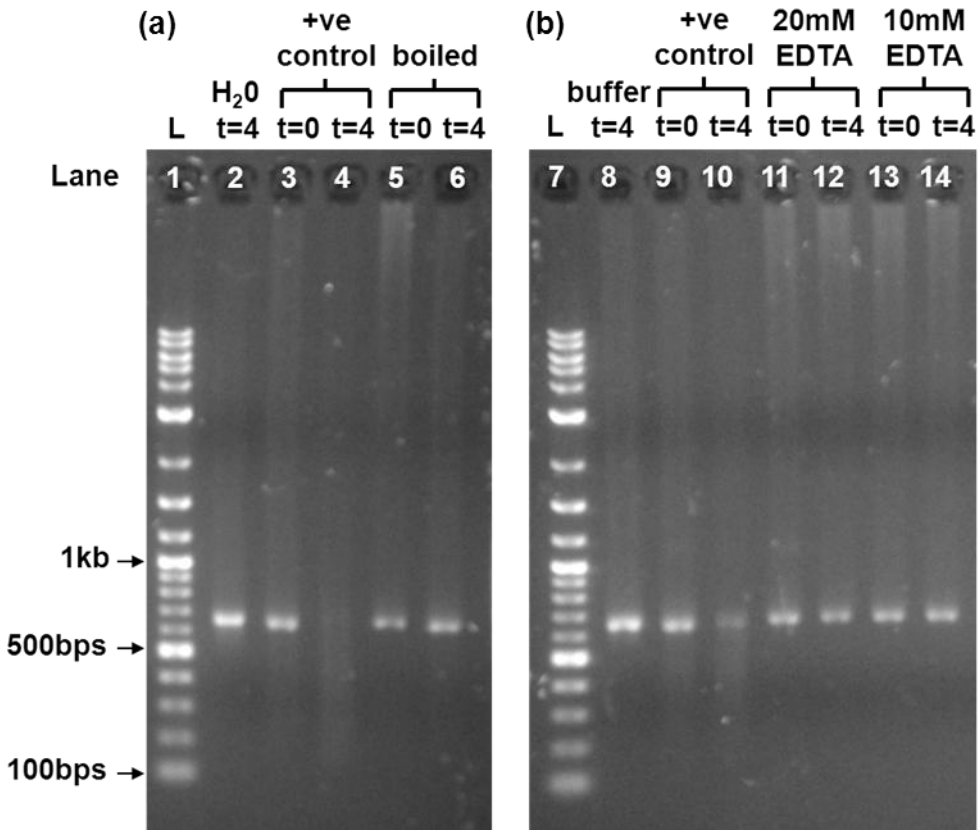


Figure 4.3: Degradation of dsRNA in *M. sexta.* In the assay dsRNA was incubated with *Manduca sexta* cell-free hemolymph for t hours after which it was recovered using an RNeasy kit (Qiagen) and run on a 1% agarose EtBr gel. In gel **(a)** lane 1 contains a DNA ladder, lane 2 contains dsRNA incubated with DEPC-treated water for 4 hours, lanes 3&4 contain dsRNA incubated with cell-free hemolymph for 0&4 hours respectively and lanes 5&6 contain dsRNA incubated with cell-free hemolymph that had previously been heated to 100°C for 5 minutes for 0&4 hours respectively. In gel **(b)** lane 7 contains a DNA ladder, lane 8 contains dsRNA incubated with MOPS buffer (40mM MOPS, pH 7.5) for 4 hours, lanes 9&10 contain dsRNA incubated with cell-free hemolymph and MOPS buffer for 0&4 hours respectively, lanes 11&12 contain dsRNA incubated with cell-free hemolymph and MOPS buffer with 20mM EDTA for 0&4 hours respectively and lanes 13&14 contain dsRNA incubated with cell-free hemolymph and MOPS buffer with 10mM EDTA for 0&4 hours respectively.

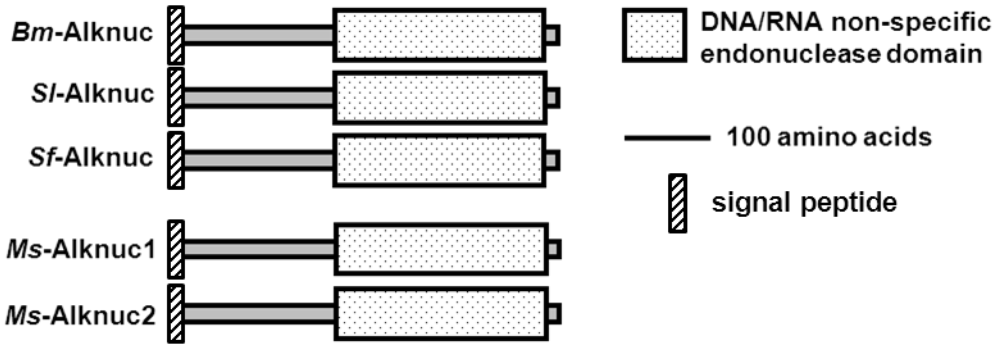
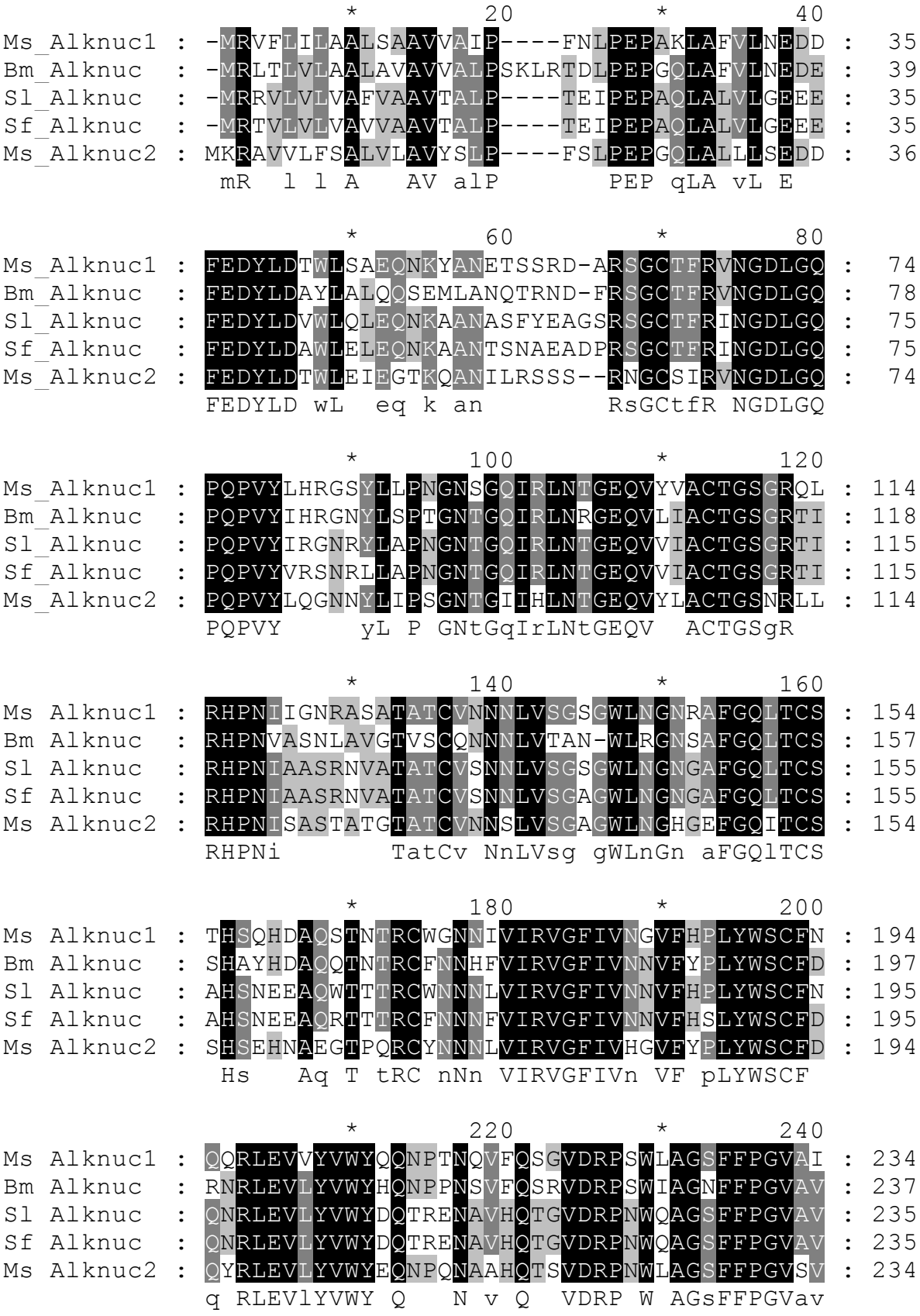


Figure 4.4: Predicted domains of *Manduca sexta* alkaline nuclease-like proteins. Domain architecture of the *Bombyx mori* (*Bm*), *Spodoptera littoralis* (*Sl)* and *Spodoptera frugiperda* (*Sf*) alkaline nuclease (Alknuc) proteins, as well as that of the two newly identified *Manduca sexta* (*Ms*) alkaline nuclease-like deduced proteins sequences (Alknuc1 & Alknuc2).


* 20 * 40
Ms_Alknuc1 : -MRVFLILAALSAAVVAIP----FNLPEPAKLAFVLNEDD : 35
Bm_Alknuc : -MRLTLVLAALAVAVVALPSKLRTDLPEPGQLAFVLNEDE : 39
Sl_Alknuc : -MRRVLVLVAFVAAVTALP----TEIPEPAQLALVLGEEE : 35
Sf_Alknuc : -MRTVLVLVAVVAAVTALP----TEIPEPAQLALVLGEEE : 35
Ms_Alknuc2 : MKRAVVLFSALVLAVYSLP----FSLPEPGQLALLLSEDD : 36
mR l l A AV alP PEP qLA vL E
* 60 * 80
Ms_Alknuc1 : FEDYLDTWLSAEQNKYANETSSRD-ARSGCTFRVNGDLGQ : 74
Bm_Alknuc : FEDYLDAYLALQQSEMLANQTRND-FRSGCTFRVNGDLGQ : 78
Sl_Alknuc : FEDYLDVWLQLEQNKAANASFYEAGSRSGCTFRINGDLGQ : 75
Sf_Alknuc : FEDYLDAWLELEQNKAANTSNAEADPRSGCTFRINGDLGQ : 75
Ms_Alknuc2 : FEDYLDTWLEIEGTKQANILRSSS--RNGCSIRVNGDLGQ : 74
FEDYLD wL eq k an RsGCtfR NGDLGQ
* 100 * 120
Ms_Alknuc1 : PQPVYLHRGSYLLPNGNSGQIRLNTGEQVYVACTGSGRQL : 114
Bm_Alknuc : PQPVYIHRGNYLSPTGNTGQIRLNRGEQVLIACTGSGRTI : 118
Sl_Alknuc : PQPVYIRGNRYLAPNGNTGQIRLNTGEQVVIACTGSGRTI : 115
Sf_Alknuc : PQPVYVRSNRLLAPNGNTGQIRLNTGEQVVIACTGSGRTI : 115
Ms_Alknuc2 : PQPVYLQGNNYLIPSGNTGIIHLNTGEQVYLACTGSNRLL : 114
PQPVY yL P GNtGqIrLNtGEQV ACTGSgR
* 140 * 160
Ms Alknuc1 : RHPNIIGNRASATATCVNNNLVSGSGWLNGNRAFGQLTCS : 154
Bm Alknuc : RHPNVASNLAVGTVSCQNNNLVTAN-WLRGNSAFGQLTCS : 157
Sl Alknuc : RHPNIAASRNVATATCVSNNLVSGSGWLNGNGAFGQLTCS : 155
Sf Alknuc : RHPNIAASRNVATATCVSNNLVSGAGWLNGNGAFGQLTCS : 155
Ms Alknuc2 : RHPNISASTATGTATCVNNSLVSGAGWLNGHGEFGQITCS : 154
RHPNi TatCv NnLVsg gWLnGn aFGQlTCS
* 180 * 200
Ms Alknuc1 : THSQHDAQSTNTRCWGNNIVIRVGFIVNGVFHPLYWSCFN : 194
Bm Alknuc : SHAYHDAQQTNTRCFNNHFVIRVGFIVNNVFYPLYWSCFD : 197
Sl Alknuc : AHSNEEAQWTTTRCWNNNLVIRVGFIVNNVFHPLYWSCFN : 195
Sf Alknuc : AHSNEEAQRTTTRCFNNNFVIRVGFIVNNVFHSLYWSCFD : 195
Ms Alknuc2 : SHSEHNAEGTPQRCYNNNLVIRVGFIVHGVFYPLYWSCFD : 194
Hs Aq T tRC nNn VIRVGFIVn VF pLYWSCF
* 220 * 240
Ms Alknuc1 : QQRLEVVYVWYQQNPTNQVFQSGVDRPSWLAGSFFPGVAI : 234
Bm Alknuc : RNRLEVLYVWYHQNPPNSVFQSRVDRPSWIAGNFFPGVAV : 237
Sl Alknuc : QNRLEVLYVWYDQTRENAVHQTGVDRPNWQAGSFFPGVAV : 235
Sf Alknuc : QNRLEVLYVWYDQTRENAVHQTGVDRPNWQAGSFFPGVAV : 235
Ms Alknuc2 : QYRLEVLYVWYEQNPQNAAHQTSVDRPNWLAGSFFPGVSV : 234
q RLEVlYVWY Q N v Q VDRP W AGsFFPGVav

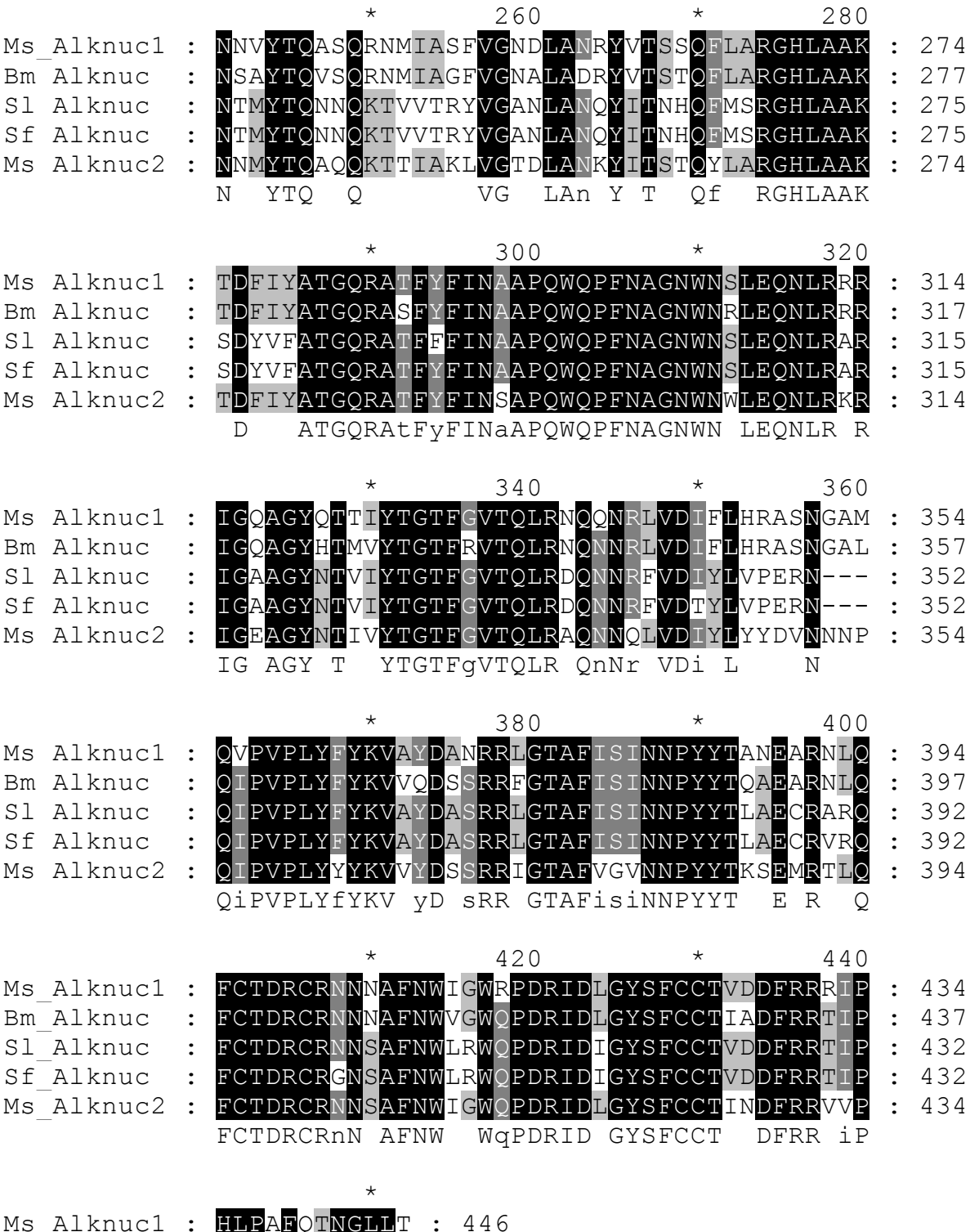

* 260 * 280
Ms_Alknuc1 : NNVYTQASQRNMIASFVGNDLANRYVTSSQFLARGHLAAK : 274
Bm_Alknuc : NSAYTQVSQRNMIAGFVGNALADRYVTSTQFLARGHLAAK : 277
Sl_Alknuc : NTMYTQNNQKTVVTRYVGANLANQYITNHQFMSRGHLAAK : 275
Sf_Alknuc : NTMYTQNNQKTVVTRYVGANLANQYITNHQFMSRGHLAAK : 275
Ms_Alknuc2 : NNMYTQAQQKTTIAKLVGTDLANKYITSTQYLARGHLAAK : 274
N YTQ Q VG LAn Y T Qf RGHLAAK
* 300 * 320
Ms_Alknuc1 : TDFIYATGQRATFYFINAAPQWQPFNAGNWNSLEQNLRRR : 314
Bm_Alknuc : TDFIYATGQRASFYFINAAPQWQPFNAGNWNRLEQNLRRR : 317
Sl_Alknuc : SDYVFATGQRATFFFINAAPQWQPFNAGNWNSLEQNLRAR : 315
Sf_Alknuc : SDYVFATGQRATFYFINAAPQWQPFNAGNWNSLEQNLRAR : 315
Ms_Alknuc2 : TDFIYATGQRATFYFINSAPQWQPFNAGNWNWLEQNLRKR : 314
D ATGQRAtFyFINaAPQWQPFNAGNWN LEQNLR R
* 340 * 360
Ms_Alknuc1 : IGQAGYQTTIYTGTFGVTQLRNQQNRLVDIFLHRASNGAM : 354
Bm_Alknuc : IGQAGYHTMVYTGTFRVTQLRNQNNRLVDIFLHRASNGAL : 357
Sl_Alknuc : IGAAGYNTVIYTGTFGVTQLRDQNNRFVDIYLVPERN--- : 352
Sf_Alknuc : IGAAGYNTVIYTGTFGVTQLRDQNNRFVDTYLVPERN--- : 352
Ms_Alknuc2 : IGEAGYNTIVYTGTFGVTQLRAQNNQLVDIYLYYDVNNNP : 354
IG AGY T YTGTFgVTQLR QnNr VDi L N
* 380 * 400
Ms_Alknuc1 : QVPVPLYFYKVAYDANRRLGTAFISINNPYYTANEARNLQ : 394
Bm_Alknuc : QIPVPLYFYKVVQDSSRRFGTAFISINNPYYTQAEARNLQ : 397
Sl_Alknuc : QIPVPLYFYKVAYDASRRLGTAFISINNPYYTLAECRARQ : 392
Sf_Alknuc : QIPVPLYFYKVAYDASRRLGTAFISINNPYYTLAECRVRQ : 392
Ms_Alknuc2 : QIPVPLYYYKVVYDSSRRIGTAFVGVNNPYYTKSEMRTLQ : 394
QiPVPLYfYKV yD sRR GTAFisiNNPYYT E R Q
* 420 * 440
Ms_Alknuc1 : FCTDRCRNNNAFNWIGWRPDRIDLGYSFCCTVDDFRRRIP : 434
Bm_Alknuc : FCTDRCRNNNAFNWVGWQPDRIDLGYSFCCTIADFRRTIP : 437
Sl_Alknuc : FCTDRCRNNSAFNWLRWQPDRIDIGYSFCCTVDDFRRTIP : 432
Sf_Alknuc : FCTDRCRGNSAFNWLRWQPDRIDIGYSFCCTVDDFRRTIP : 432
Ms_Alknuc2 : FCTDRCRNNSAFNWIGWQPDRIDLGYSFCCTINDFRRVVP : 434
FCTDRCRnN AFNW WqPDRID GYSFCCT DFRR iP
*
Ms_Alknuc1 : HLPAFQTNGLLT : 446
Bm_Alknuc : HLPAFNVNGLLT : 449
Sl_Alknuc : HLPSFSTNGLLA : 444
Sf_Alknuc : HLPSFSTNGLLA : 444
Ms_Alknuc2 : HLPSFTVNGLLS : 446
HLP F NGLL

Figure 4.5: Multiple alignment of the two newly identified *Manduca sexta* (*Ms*) alkaline nuclease deduced amino acid sequences (Alknuc1 & Alknuc2) with the *Bombyx mori* (*Bm*), *Spodoptera littoralis* (*Sl)* and *Spodoptera frugiperda* (*Sf*) alkaline nuclease (Alknuc) amino acid sequences. Where amino acid residues are shared by all five sequences they are shaded in black, where there is consensus between four of the five sequences the residues are shaded in dark grey and where there is consensus between three of the five sequences the residues are shaded in light grey. The consensus sequence is included below the alignment. Numbers and asterisks above the alignment and numbers to the right of the alignment indicate position along the amino acid sequences.

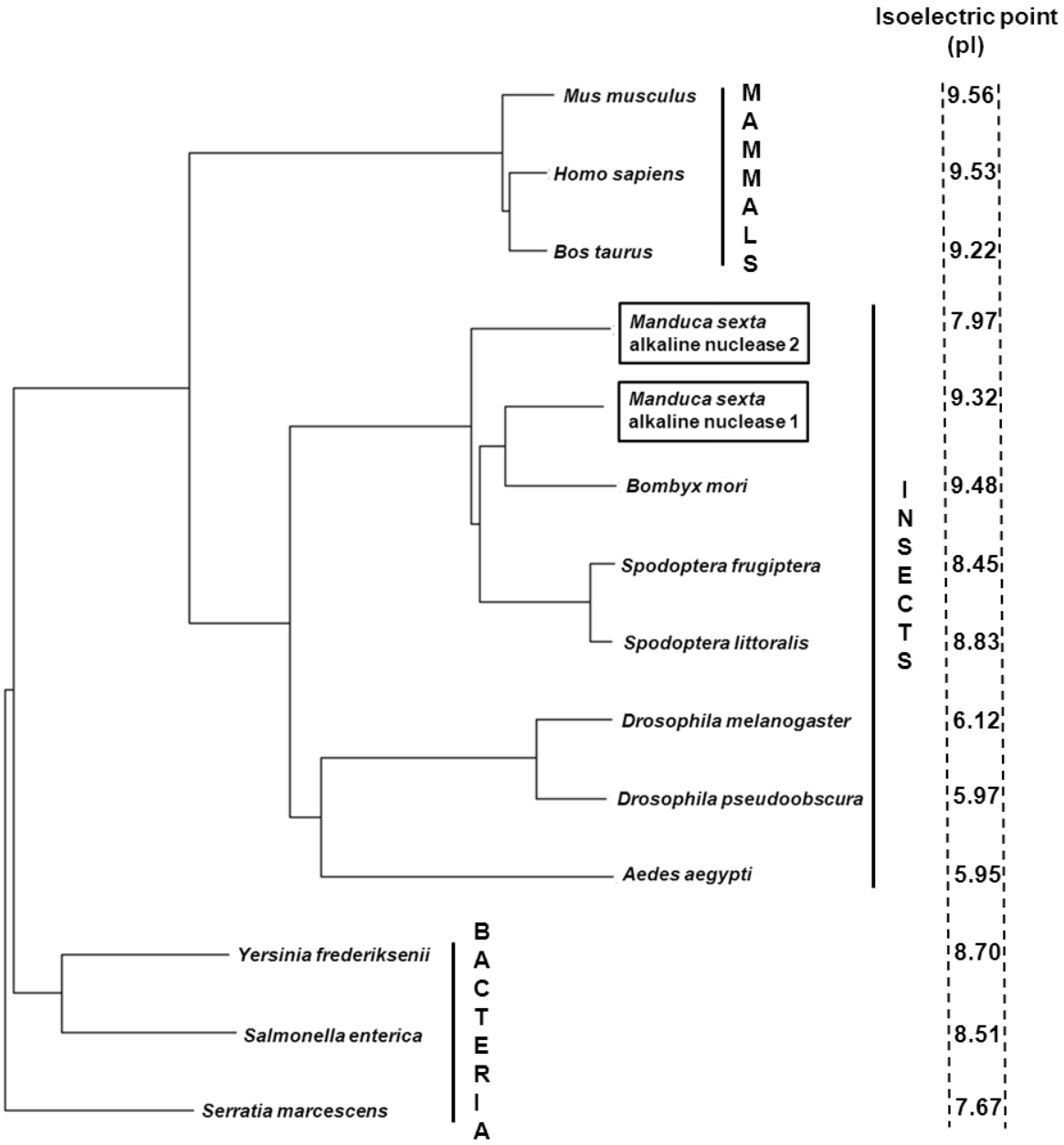
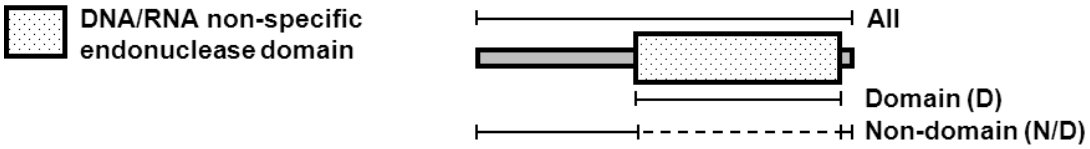


Figure 4.6: Neighbour-joining tree showing the phylogenetic relationship of the newly identified *Manduca sexta* alkaline nuclease deduced amino acid sequences with DNA/RNA non-specific nucleases. The amino acid sequences used for the analysis were from GenBank under following accession numbers: *Bombyx mori* (BAF33251.1); *Spodoptera littoralis* (CAR92522.1); *Spodoptera frugiptera* (CAR92521.1); *Serratia marscesence* (CAA00836); *Salmonella enterica*, putative DNA/RNA non-specific endonuclease (AAL91099); *Yersinia frederiksenii*, DNA/RNA endonuclease G (ZP_00829494); *Aedes aegypti*, putative deoxyribonuclease I (EAT42072); *Drosophila melanogaster*, CG3819-PA (AAF49206); *Drosophila pseudoobscura*, GA17708-PA (EAL30415); *Mus musculus*, endonuclease G (NP_031957); *Homo sapiens*, endonuclease G (AAH16351); *Bos taurus*, endonuclease G (P38447). The tree was rooted using the *Serratia* sequence as an outgroup. The predicted isoelectric point (pI) of the protein sequences (calculated using the tool found at http://expasy.org/tools/protparam.html) is included in a column on the right.



	M. sexta AIknuc1			*M. sexta* AIknuc2			*B. mori*			*S. littoralis*			*S. frugiperda*		
	All	D	N/D	All	D	N/D	All	D	N/D	All	D	N/D	All	D	N/D
M. sexta AIknuc1	-			69	75	62	76	84	65	72	73	69	71	74	68
M. sexta AIknuc2	-			-			66	75	54	68	73	62	68	73	61
B. mori	-			-			-			68	72	63	68	71	64
S. littoralis	-			-			-			-			95	97	91
S. frugiperda	-			-			-			-			-		

Figure 4.7: Amino acid identity scores arising from the alignment of the two newly identified *Manduca sexta* alkaline nuclease deduced amino acid sequences with the *B. mori*, *S. littoralis* and *S. frugiperda* alkaline nuclease amino acid sequences. Scores are given for the entire length of the protein (All), for alignment of the DNA/RNA non-specific endonuclease domains only (D) and for alignment of the regions not coding for this domain only (N/D).

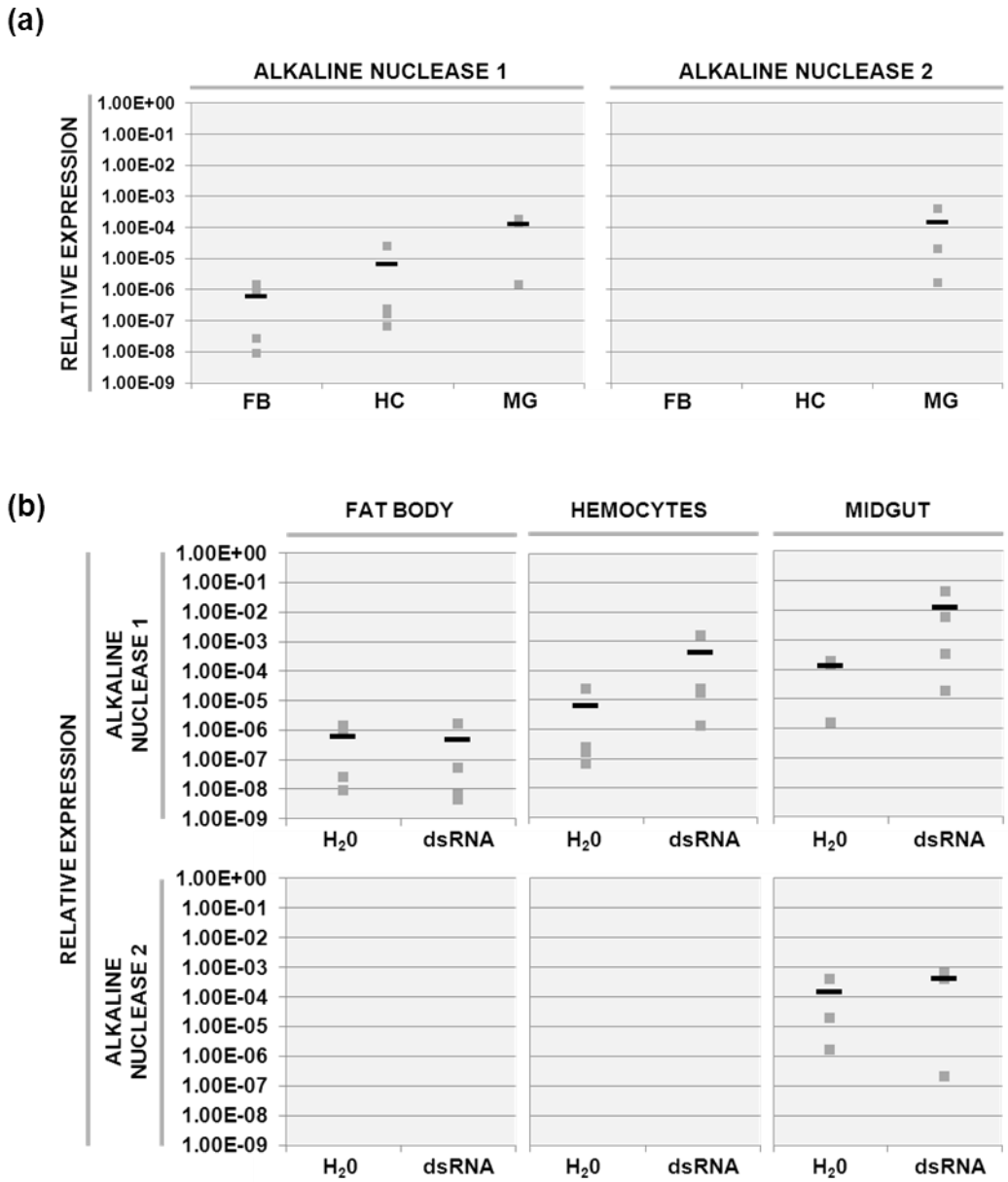


Figure 4.8: Relative expression of the newly identified *Manduca sexta* alkaline nuclease 1&2 mRNA. (a) mRNA levels in control water-injected (H_20) insects and **(b)** in insects injected with eGFP dsRNA (n=4). Newly ecdysed fifth instar larvae were injected with water or 1 µg dsRNA. Six hours post injection fat body, hemocytes and midgut were dissected and subjected to q-RT-PCR to quantify mRNA levels. The values for the four replicates are shown as grey squares and the mean is depicted with a black dash.

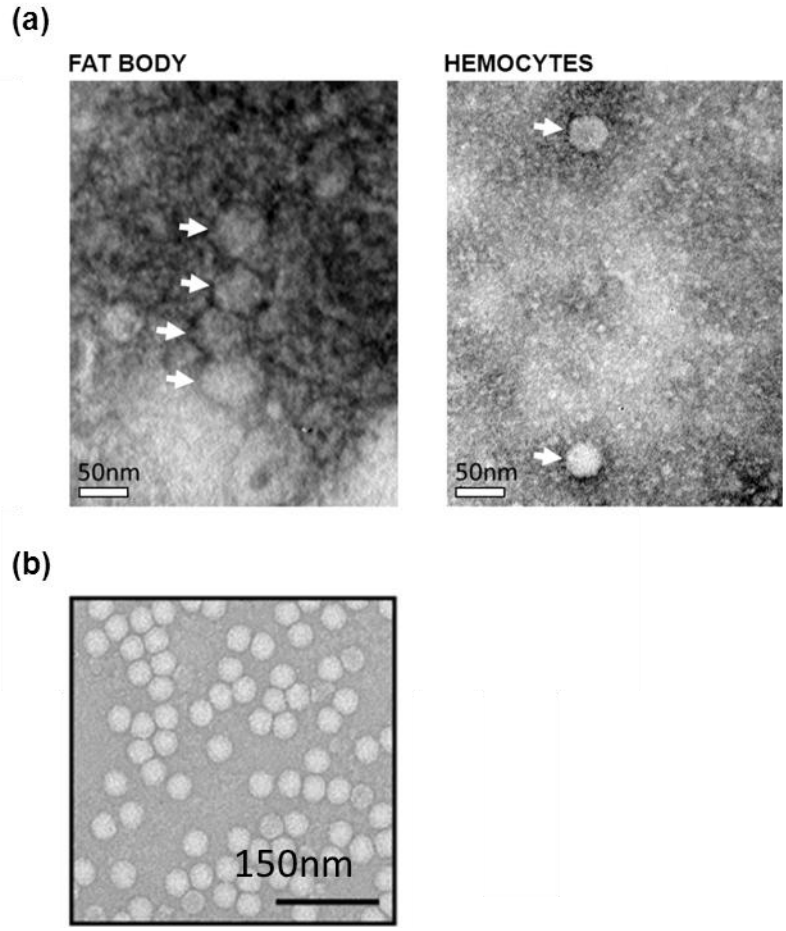


Figure 4.9: Detection of viral particles in *M. sexta* using electron microscopy. (a) Transmission electron microscopy (TEM) images following negative staining of *M. sexta* fat body and hemocytes in 2% aqueous uranyl oxylate. Fat body tissue and hemocytes were dissected from newly emerged *M. sexta* larvae, fixed in 2.5% glutaraldehyde, 2% paraformaldehyde and 2.5mM $CaCl_2$ in 0.1M sodium cacodylate buffer (pH 7.4), washed in 0.1M sodium cacodylate and incubated for 1 hour at room temperature with 1% osmium tetroxide and 1% potassium ferrocyanide. The tissues were then washed in distilled water, incubated with 2% aqueous uranyl oxylate for 1 hour in the dark, dehydrated progressively in acetone and infiltrated in Spurrs Epoxy Resin overnight at room temperature on a stirrer. Tissue samples were embedded in fresh Spurrs Epoxy Resin, polymerised in an oven at 70°C for seven hours and the resin blocks sectioned at 60nm on a Reichert OMU3 microtome using glass knives (TAAB) and collected onto copper/palladium grids. **(b)** Flock House Virus (FHV) particles imaged using similar techniques by Walukiewicz *et al* (2006).

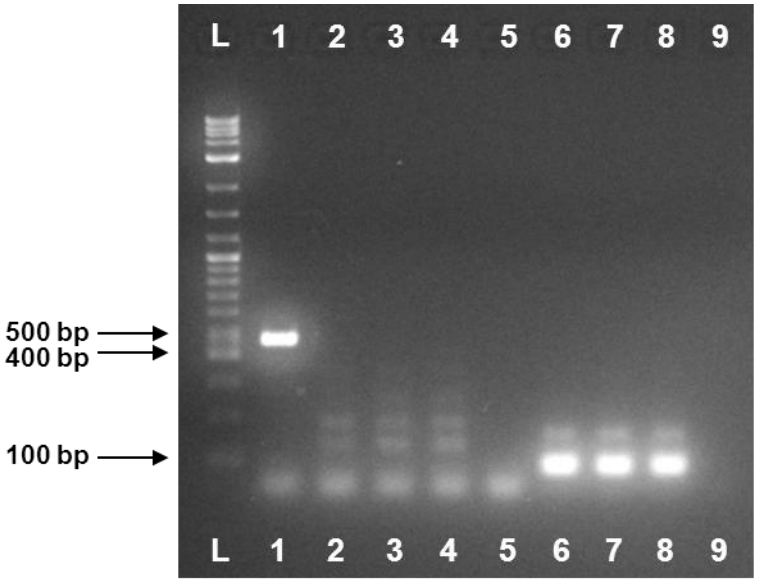


Figure 4.10: **PCR detection of mycoplasma species.** Universal mycoplasma primers (F1: 5'-ACA CCA TGG GAG (C/T)TG GTA AT-3' & R1: 5'-CTT C(A/T)T CGA CTT (C/T)CA GAC CCA AGG CAT-3') were used to amplify genomic DNA from 1. *Drosophila melanogaster* "Red" adult females, 2. first instar *M. sexta* larvae, 3. *M. sexta* third instar larvae & 4. fat body from fifth instar larvae. Lane 5 contains a non-template control. Primers for a *Manduca sexta* internal control gene, RPS-3, were used in a PCR to confirm the integrity of genomic DNA in 6. *M. sexta* hatchlings, 7. *M. sexta* third instar larvae & 8. fat body from fifth instar larva. Lane 9 contains a non-template control.

CHAPTER 5: THE UPTAKE OF DOUBLE-STRANDED RNA INTO INSECT TISSUE DURING *IN VIVO* RNA INTERFERENCE EXPERIMENTS

5.1 Introduction

One potential explanation for the observed insensitivity of some insect species to *in vivo* RNA interference (Terenius *et al.*, 2011) is that uptake of dsRNA into cells in those insects is not sufficient to initiate a knockdown of gene expression. Since dsRNA processing and the dsRNA-mediated targeting and degradation of mRNA are cellular processes (Zamore *et al.*, 2000; Hammond *et al.*, 2000; Elbashir *et al.*, 2001*a*, 2001*b*; Nykänen *et al.*, 2001) it follows that dsRNA must be taken up into cells in order for a knockdown to occur. The internalisation of dsRNA is, therefore, a potentially limiting step in the RNAi pathway.

Experiments conducted in cultured insect cells have revealed that uptake of dsRNA can be a limiting factor in RNAi experiments. Beck & Strand (2003, 2005) observed that Hi5 cells (derived from *Trichoplusia ni*), which are deficient in RNAi when dsRNA is provided in the cell culture media, are not able to efficiently internalise dsRNA from the media. Interestingly, very efficient intracellular RNAi was achieved in the same cells when the dsRNA was introduced together with a transfection reagent, indicating that uptake was the limiting factor in this system. Johnson *et al.* (2010) reported similar results in the UGA-CiE1 cell line (derived from *Chrysodeixis includens*); they found that the efficiency of RNAi in the cells was greatly improved by transfection of the dsRNA.

It is thus evident from experiments conducted in cell culture systems that some cell types are deficient in dsRNA uptake, and that this deficiency is responsible for their lack of an effective environmental RNAi response. However, since cultured cells are likely to behave differently to cells situated in the complex structural and chemical environment of an organism, it does not necessarily follow that dsRNA uptake can be deficient in cells *in situ.* One indication that dsRNA uptake can be inadequate in a whole organism comes from the observation that, whilst RNAi is extremely efficient in *Drosophila melanogaster* cells when the RNAi trigger (dsRNA) is introduced directly into the cells by the expression of transgenic hairpin sequences, it is ineffective in most larval tissues following the experimental introduction of dsRNA *in vivo* (Miller *et al.*, 2008). These circumstances suggest that larval *D. melanogaster* tissues are defective in dsRNA uptake.

In addition to the internalisation of dsRNA, the amplification and spread of the dsRNA signal within target tissues (a process termed “systemic RNA”) may also play an important

role in governing the success of an RNAi experiment. In the nematode worm *Caenorhabditis elegans* a transmembrane channel protein, encoded by the *systemic RNA interference deficient-1* (*sid-1*) gene, is necessary for systemic RNAi (Winston *et al.*, 2002) and functions to enable the uptake of dsRNA into cells (Feinberg & Hunter, 2003). As yet the role of the SID-1 channel protein in systemic RNAi has not been clearly demonstrated in insects. Evidence for a role of SID-1-like proteins in insect RNAi comes from the observation that *Drosophila melanogaster* flies (in which SID-1-like genes appear to be absent; Roignant *et al.*, 2003) exhibit deficiency in RNAi when injected with dsRNA (Miller *et al.*, 2008) or fed yeast-expressed dsRNA (Gura, 2000).

The study reported in this Chapter aims to identify whether inadequate uptake of dsRNA into cells is responsible for the variable response to RNAi observed in *Manduca sexta* (see Chapter 3). Three assays were developed in order to quantify uptake of injected dsRNA into *M. sexta* tissues *in vivo*. In addition, the study addresses the question of whether *M. sexta* encodes a SID-1-like gene. These results may shed light on whether uptake of dsRNA into cells, or spreading of the dsRNA signal, could be limiting factors during *in vivo* RNAi experiments.

5.2 Results

5.2.1 Detection of dsRNA in Manduca sexta *tissue using q-RT-PCR*

In order to study the uptake of dsRNA into *M. sexta* tissue an assay based on the detection by quantitative reverse transcription PCR (q-RT-PCR) of injected dsRNA was developed. Uptake of injected eGFP dsRNA into tissue was quantified using a modified q-RT-PCR protocol which has already been described in chapter 4, section 4.2.1. Figure 5.1a is a reproduction of Figure 4.1a and shows the results of the calibration of the assay. The equation derived from the calibration assay was used to quantitate uptake of dsRNA into *M. sexta* tissue. Following injection of eGFP dsRNA into newly emerged fifth instar larvae and dissection of tissue (or collection of hemocytes; see section 2.2 for details) total RNA was extracted from a known mass of tissue (for analysis of uptake into fat body and midgut) or from a known volume of hemolymph (for analysis of uptake into hemocytes). An aliquot of total RNA was used for q-RT-PCR and the quantity of dsRNA per microlitre of hemolymph (for hemocytes) or per microgram of tissue (for fat body and midgut tissue) was calculated by scaling up the quantity of dsRNA represented by the qPCR output (determined using the calibration equation) according to the appropriate dilution factors.

Using the above described methods, dsRNA was detected in fat body and midgut tissue and in hemocytes six hours post injection with 4 µg dsRNA, in quantities that were significantly above the detection limit (Figure 5.1b). In contrast dsRNA could not be detected in tissue following injection with a DEPC-treated water control (Figure 5.1b; any signal obtained from the assay fell below the detection limit). More dsRNA was detected in fat body than in midgut tissue and this difference was near-significant (t-test; t=-2.380, p=0.05476).

It is straightforward to compare the presence of dsRNA in in midgut and fat body tissue given the similarity of the methods used to estimate the quantity of dsRNA. In contrast, it is not easy to make sensible comparisons of uptake into fat body or midgut with uptake into hemocytes, because the quantity of dsRNA detected in hemocyte tissue was expressed using different units (quantity of dsRNA per microlitre of hemolymph rather than per microgram of tissue). In order to make a sensible comparison we need to be able to estimate the mass of tissue present in a microlitre of hemolymph. Following centrifugation of 1 ml hemolymph, freshly drawn from a fifth instar *Manduca sexta* caterpillar, the volume of the packed cellular pellet can be roughly estimated as in the order of 1 µl (i.e. equivalent to about 1 mg in weight). This figure has not been accurately estimated, but it is very unlikely to be in error by more than 1 order of magnitude, either too low or too high. The mass of tissue present in 1 µl of fresh hemolymph can therefore be estimated to be equivalent to 1 µg (1000 times less than in 1 ml hemolymph). The data presented in Figure 5.1b are, therefore, roughly comparable (since 1 µl of fresh hemolymph will contain approximately 1 µg of cells) and indicate that uptake of dsRNA into hemocytes is considerable higher than into midgut and fat body tissue, when calculated on a tissue weight basis (t-test on log transformed data; midgut t=12.31, p=1.75e-05, fat body t=8.757, p<0.001).

5.2.2 Detection of dsRNA in M. sexta *hemocytes using flow cytometry*

Following the detection of injected dsRNA in *M. sexta* larval tissue by q-RT-PCR an alternative means of detection was sought in order to confirm and further study the uptake of dsRNA into cells. An assay was developed whereby fluorescent dsRNA injected into the hemocoel of the larva was later detected within hemocytes by flow cytometry. Hemocytes were chosen for further study because the results from the q-RT-PCR assay indicate that hemocytes are responsible for a large degree of uptake into larval tissue, and because hemocyte cells are relatively easy to separate and analyse using flow cytometry.

When hemocytes were isolated from *M. sexta* larvae one hour after injection with FITC-labelled dsRNA the properties of the cells as determined by flow cytometry were

significantly different to the properties of hemocytes from control insects (injected with water). Analysis of hemocytes from control insects revealed that they make up a discrete, single population when cell fluorescence (measured in the FITC channel) is plotted against either of two measures of the optical properties of the cells (forward scatter or side scatter of visible light; Figure 5.2 i a&b, and appendix Figure A1). By comparison, in the equivalent plots of hemocytes from insects injected with FITC-dsRNA, an additional population of cells, with higher fluorescence, is apparent (Figure 5.2 ii-iv a&b and appendix Figure A1). The presence of a cell population with markedly increased fluorescence in hemocytes originating from insects injected with labelled dsRNA (compared to those from insects receiving the control injection) is further revealed when the intensity of the fluorescent signal (FITC) in the cells is plotted as a histogram (Figure 5.2 i-iv, c&d and appendix Figure A1).

The data presented so far indicate that at least some of the fluorescent signal incorporated in the introduced dsRNA becomes associated with some hemocytes. Close study of the scatter diagrams, but more particularly the histograms presented in Figure 5.2, indicates that the association of hemocytes with dsRNA may be dependent on the dose of dsRNA injected. Plotting the mean fluorescence intensity of the hemocyte populations for the different injection treatments (the mean of the mean fluorescence of the 30,000 cells analysed per sample for the five replicates in the H_20, 1 microgram and 5 microgram injection treatments and for two replicates in the 10 microgram injection treatment) not only confirms that cells from insects injected with dsRNA have higher associated fluorescence but also indicates that fluorescence is dependent on the dose of dsRNA (Figure 5.2e). Indeed, plotting fluorescence versus the dose of dsRNA in a scatter plot shows that as the dose of dsRNA increases there is an exponential increase in mean fluorescence (Figure 5.2f). Furthermore, linear regression analysis shows that there is a significant positive relationship between the dose of dsRNA and the log transformed mean fluorescence of the cell population (Figure 5.2g; $t=8.598$, $p<0.001$, $y=0.057697x+1.626459$).

5.2.3 Flow cytometry reveals that dsRNA is taken up into a subset of hemocytes

Although analysis of the cell population as a whole reveals an increase in mean fluorescence in hemocytes from dsRNA-injected insects (Figure 5.2), indicating association of the hemocyte population with dsRNA, it is clear that in reality a very small proportion of the hemocytes are implicated in this association. Gating of the hemocytes into three cell populations based on their FITC (fluorescence) value allowed further analysis of the cells. Population 1 (P1) was defined as including cells with low fluorescence (more than 99% of

cells from the control injection fall within this category), Population 2 (P2) contains cells with medium fluorescence and Population 3 (P3) is made up of cells with high fluorescence (none of the cells from the control injection are in this category) (Figure 5.3a).

Plotting the percentage of cells from the entire parent population which fall into the three fluorescence categories shows that there is a dose-dependent decrease in the number of cells in the low fluorescence category (P1) and a dose-dependent increase in the number of cells in the medium (P2) and high (P3) fluorescence categories following injection with fluorescently labelled dsRNA (Figure 5.3b). However, very few cells, even in insects injected with the largest dose of dsRNA, are found in the medium and high fluorescence populations (in other words few cells show increased fluorescence). When insects were injected with a 10 microgram dose of dsRNA only 2.9% of cells (870/30,000) fell into P2 (had medium fluorescence) and 2.9% of cells (870/30,000) fell into P3 (had high fluorescence). This means that less than 6% of the hemocytes appear to be associated with the fluorescent label incorporated in the introduced dsRNA molecules.

In order to speculate as to the identity of the small subset of cells observed to be associated with dsRNA, the properties of cells exhibiting high fluorescence were further analysed. Cells derived from insects injected with a 5 microgram dose of dsRNA were chosen for analysis, since this treatment represented a sufficiently high dose and contained a reasonable number of replicates (n=5). Three sources of data describing the properties of hemocytes were gathered during the flow cytometry experiment. The first was the FITC-channel fluorescence of the cells, the second was the amount of light scattered forward when the cell passed the laser, which gives an indication of cell size (bigger cells scatter more light forward and have a greater forward scatter value (FSC-H) (Latimer, 1982; Shapiro, 1995) and the third was the amount of light scattered sideways when the cell passed the laser, which gives an indication of the granularity of the cells (more granular cells scatter more light sideways and have a greater side scatter value (SSC-A) (Latimer, 1982; Shapiro, 1995).

Since the cells were gated into populations based on their fluorescence (Figure 5.3a) it follows that the mean fluorescence for the different populations varies. Indeed, the three populations differ greatly in their fluorescence (cells in P1 have a mean FITC value of 44.8, compared to values of 491.8 and 2537.2 in P2 and P3 respectively; Figure 5.4a). Fluorescence in P2 and P3 is significantly greater than in P1 (t-tests; $t=19.556$, $p<0.001$ and $t=21.474$, $p<0.001$ respectively).

The three populations also vary in their mean forward and side scatter values. Cells in P2 and P3 (with medium and high fluorescence) scatter significantly less light forward (have reduced forward scatter; FSC-H) compared to cells in P1 (Figure 5.4b; t-tests: P2 $t=-4.399$,

p=0.00229, P3 t=-14.17, p<0.001). In the case of side-scatter (SSC-A), cells in P2 do not scatter light sideways significantly differently from cells in P1 (Figure 5.4c; t=0.846, p=0.422). However, cells in P3 do scatter significantly less light sideways than cells in P1 (Figure 5.4c; t=-12.36, p<0.001). The fact that cells with increased fluorescence display, in general, lower forward scatter and side scatter compared to cells in the main cell population is further apparent when viewing plots of side scatter and forward scatter versus fluorescence (FITC) (Figure 5.2a&b). The majority of cells with high fluorescence clearly have lower side-scatter and forward-scatter values than those in the main cell population. These results suggest that hemocytes displaying higher fluorescence (that are therefore likely to contain internalised dsRNA) are relatively small in size and low in granularity.

5.2.4 Confocal microscopy confirms uptake into hemocytes

In order to verify that the flow cytometry data represent internalisation of dsRNA into *M. sexta* hemocytes and to explore whether individual subpopulations of hemocytes are responsible for the majority of dsRNA uptake (as indicated by the flow cytometry data) a microscopy approach was employed. DEPC-treated water containing 10 µg fluorescein-labeled dsRNA was injected into newly emerged fifth instar larvae, from which hemocytes were dissected, adhered to coverslips, fixed and stained with TRITC-linked phalloidin (which binds F-actin) and imaged using confocal microscopy. No green fluorescent signal was detected in hemocytes derived from control insects injected with DEPC-treated water (Figure 5.5a), whilst there was significant co-localisation of green fluorescence (incorporated in the introduced dsRNA) with red fluorescence (indicating actin components of the hemocytes) in hemocyte preparations from insects injected with fluorescein-labeled dsRNA and incubated for 1 hour (Figure 5.5d & f). An orthogonal projection of a series of images taken at different optical depths (a z-stack; Figure 5.5h) shows that the green fluorescence is located internally within the hemocyte cell (the cell type illustrate in the figure is a plasmatocyte). In the image the intense green signal is surrounded in both planes by red, indicating that dsRNA had been internalised. Internalisation of dsRNA was also observed in an *in vitro* variant of the uptake assay (Figure 5.6 a, d, f & h).

In order to establish whether the observed uptake of dsRNA into hemocytes was taking place via an active process, a treatment where the injected insects were incubated at a cooler temperature was included. When the fluorescent-dsRNA-injected insects were incubated for 60 minutes at 4°C (rather than at room temperature) there was still appreciable uptake of dsRNA into plasmatocytes (Figure 5.5e). If uptake of dsRNA into hemocytes was an active process we would expect that lowering the temperature would slow down the rate of uptake

or even suppress uptake completely. Whilst it could be argued that the cells in the 4°C treatment show slightly reduced levels of uptake (compared to the cells from the room temperature treatment; Figure 5.5d), these conditions fail to totally suppress uptake into plasmatocytes. This may indicate that the uptake process does not depend on active cellular processes. However, another possibility that must be considered is that this treatment was insufficient to prevent such active processes (possible the insects were not cooled sufficiently or rapidly enough). Finally, it is possible that at 4°C dsRNA import could still be an active process, depending on the dymanics and saturation of the relevant pathway.

5.2.5 Confocal microscopy indicates that the majority of dsRNA is taken up into plasmatocytes

Confocal microscopy allowed the identification of different hemocytes classes, which were grouped according to the criteria adopted by Dean *et al.* (2004). Plasmatocytes (PL) were defined as being pleiomorphic spreading cells, commonly possessing several extending filopodia and being relatively flat, whilst granular cells (GR) were defined as being rounded with a small diameter (Dean *et al.*, 2004). The adherent hemocytes in the experiments presented here were identified as being either plasmatocytes or granular cells based on these criteria (Figure 5.5a & Figure 5.6a). Granular cells appeared smaller in diameter than plasmatocytes and confocal optical sections showed that plasmatocytes were thin in comparison with rounder granular cells. Although other hemocytes types exist in *M. sexta* (such as oenocytoids, spherulocytes and prohemocytes; Dean *et al.*, 2004), these were not observed in the monolayer preparations.

The *in vivo* dsRNA uptake experiment found that dsRNA is internalised into plasmatocytes, with little uptake into granular cells (at 60 minutes post injection; Figure 5.5 d, f & g), indicating that plasmatocytes preferentially take up dsRNA. Within the plasmatocytes the internalised label appears to be concentrated and localised in structures located in the perinuclear region, which are likely to be vesicles of some kind. In contradiction with the data from the *in vivo* uptake experiment, when uptake into hemocytes was assayed *in vitro* the majority of uptake occurred in granular cells rather than plasmatocytes (at 60 minutes incubation time; Figure 5.6 d, f & g). There appeared to be more uptake into granular cells *in vitro* than observed *in vivo* (compare Figures 5.5g & 5.6g) and less uptake into plasmatocytes *in vitro* (compare Figures 5.5d & 5.6d). The intensity of the plasmatocytes-located dsRNA-fluorescein signal in the orthogonal projections (Figures 5.5h & 5.6h) appeared to be greater in the *in vivo* experiment (Figure 5.6h).

5.2.6 Uptake of dsRNA into hemocytes is rapid

Quantitative reverse transcription PCR (q-RT-PCR), flow cytometry and confocal microscopy were employed to investigate the dynamics of dsRNA uptake into insect tissue. A time course experiment utilising the newly developed q-RT-PCR protocol indicated that uptake into tissue occurred rapidly, with detection of the dsRNA signal at a maximum at 1 hour post injection in hemocytes and midgut and at 6 hours post injection in fat body (Figure 5.7). Following these initial peaks in dsRNA levels, the presence of dsRNA in tissue decreased rapidly with levels falling near to or below the detection limit of the assay by 18 hours post dsRNA injection.

Flow cytometry experiments varying the time between injection of fluorescently-labelled dsRNA and dissection of the hemolymph revealed that, whilst a marked increase in the fluorescence of some cells was observed when the time of incubation was one hour (Figure 5.2), no difference in the fluorescence of cells was observed when incubation occurred for longer periods of time (data not shown). This indicates that under these conditions the FITC-dsRNA is taken up into cells rather quickly (a time scale of tens of minutes), following which it either loses its fluorescent label, or is completely destroyed. A time course experiment revealed that uptake is, in fact, occurring very rapidly, with cells with higher fluorescence appearing not long after dsRNA injection (Figure 5.8 a-d) and with mean fluorescence of the dissected cells peaking 30 minutes after injection with dsRNA (Figure 5.9a).

Curiously, near significantly increased levels of fluorescence relative to the control were observed in cells from FITC-dsRNA-injected insects at time zero (when the hemolymph was dissected immediately following injection with FITC-dsRNA; Figure 5.9a, $t=2.984$, $p=0.0964$). There are several potential explanations for this observation; the first being that uptake of labelled dsRNA into cells takes place so rapidly that a degree of uptake had already occurred before the hemolymph could be removed from the insect. Another explanation is that dsRNA uptake was able to take place after the hemolymph had been dissected from the insect, but before washing. The third and final explanation is that the increased fluorescence observed in cells at time zero represents attachment of the dsRNA to the outer surface of the hemocytes, and not its internalisation into these cells.

It is interesting to note that, whilst the mean fluorescence of the cells is greater than the control at time zero, study of the histogram of fluorescence (FITC-A) reveals that this heightened fluorescence is caused almost entirely by an increase in the number of cells with medium fluorescence rather than an increase in the number of cells with high fluorescence (Figure 5.9b). This observation was confirmed when the cells were once again gated into

three populations based on their fluorescence (P1, P2 & P3 being populations containing cells with low, medium and high fluorescence respectively). The number of cells in population 2 (with medium fluorescence) is highest between 0 and 30 minutes post dsRNA injection, after which there are very few cells in the population, whereas the maximum number of cells in population 3 (with high fluorescence) occurs at 30 minutes post dsRNA injection, and is considerably lower before and after this time point (Figure 5.9c). These observations lead to the hypothesis that cells displaying a medium level of fluorescence have labelled dsRNA attached to their surface, whereas cells that are highly fluorescent have internalised dsRNA.

Confocal microscopy experiments also found that dsRNA was rapidly internalised into hemocytes. Study of *in vivo* uptake found that a small quantity of fluorescent dsRNA was present in plasmatocytes just 5 minutes after dsRNA injection (Figure 5.5b). At 20 minutes post incubation a little more dsRNA was detected (Figure 5.5c), and considerably more dsRNA was found in plasmatocytes 60 minutes post dsRNA injection (Figures 5.5d & f). Uptake of dsRNA *in vitro* followed similar uptake dynamics; substantial levels of dsRNA were detected in plasmatocytes and granular cells after 60 minutes incubation (Figure 5.6d, f & g), although little uptake was observed at the preceding time points (5 minutes and 20 minutes; Figures 5.6b & c). Attachment of dsRNA to the cell surface at early time points was not observed. Therefore, whilst the confocal microscopy data are consistent with the hypothesis generated to explain the flow cytometry data (that the fluorescent signal detected at 0-15 minutes post dsRNA injection represents attachment of the dsRNA to the outer surface of the hemocytes, with uptake of dsRNA occurring at around 30 minutes post injection) because the dsRNA uptake appears to occur subsequent to 30 minutes post injection, there is no direct evidence for attachment of the dsRNA to the surface of the hemocytes.

5.2.7 Manduca sexta *has a* SID-1-*like gene*

Using degenerate primers based on conserved regions of insect deduced amino acid sequences (as used by Tian *et al.*, 2009) a partial SID-1-like sequence (183 bp) was obtained from *M. sexta* fat body by RT-PCR (Figure 5.10a). The cloned sequence contained an open reading frame encoding 61 amino acid residues and the protein sequence was found to exhibit a reasonable degree of amino acid identity with the *C. elegans* SID-1 protein (Figure 5.10b; 29% identity; NCBI Accession NP_504372.2). Phylogenetic analysis placed the *M. sexta* SID-1 sequence in a clade with the *Bombyx mori* SID-1-like protein 3 (Figure 5.11a), indicating that the *Manduca sexta* protein is most similar to this protein. Indeed, the *M. sexta*

deduced protein sequence shows high amino acid identity with the *Bombyx mori* SID-1-like protein 3 (Figure 5.11b; 87%; BAF95806.1) and considerably less identity with the SID-1-like protein 1 (53%; BAF95805.1) and the SID-1-like protein 2 (49%; BAF95807.1) from the same species.

5.2.8 M. sexta *SID-1 is expressed in larval tissue*

Quantitative reverse transcription PCR (q-RT-PCR) analysis found that the newly identified *M. sexta* SID-1 gene was expressed in fat body and midgut tissue and in hemocytes (Figure 5.12a). Expression levels in hemocytes appeared to be lower than levels in fat body and midgut. Statistical analysis found tissue to be a marginally significant predictor of SID-1 transcript levels (ANOVA; $F=4.2589$, $p=0.04994$) and post hoc pairwise comparisons (t-tests) found that expression levels of SID-1 were considerably lower in hemocytes than in fat body ($t=-2.979$, $p=0.02467$) and midgut ($t=-2.714$, $p=0.03491$) tissue (with no significance difference in expression between fat body and midgut tissue; $t=-0.476$, $p=0.65085$**)**. Nevertheless, when Holm's sequential Bonferroni correction (Holm, 1979; Ludbrook, 1998) was applied to the tests to reduce the risk of Type I error associated with multiple comparisons, there was found to be no significant difference in the levels of expression of SID-1 in hemocytes compared with the expression levels in fat body ($p=0.07401$) and midgut ($p=0.06982$) tissue.

In addition to the resting levels of SID-1, the expression levels of this gene under conditions of dsRNA exposure may differ in different tissues and this could be responsible for the different degrees of uptake observed in different tissues. Comparison of transcript levels in insects injected with a water control or with dsRNA revealed that there was no significance difference in the expression of SID-1 in fat body ($t=0.477$, $p=0.6503$), midgut ($t=0.632$, $p=0.551$) and hemocytes ($t=0.679$, $p=0.522$), indicating that there was no upregulation of SID-1 gene expression in response to dsRNA (Figure 5.12b). Furthermore, when the expression of SID-1 in different tissues in insects injected with dsRNA was compared it was found that there was no significance in expression levels (ANOVA; $F=1.2843$, $p=0.3231$) and post hoc t-tests found no significant pairwise differences in gene expression (hemocyte-fat body $t=2.223$, $p=0.06794$; hemocyte-midgut $t=1.320$, $p=0.235$; fat body-midgut $t=0.129$ $p=0.9019$). These expression data therefore only represent weak evidence for a difference in expression of SID-1 in different tissues. Not only were the results of statistical tests non-significant, but there was extensive variability in the levels of expression of SID-1 in fat body and midgut, making sensible comparisons of expression levels difficult.

5.3 Discussion

5.3.1 Is dsRNA taken up into Manduca sexta *cells?*

This chapter is concerned with the uptake of dsRNA into insect tissue, and the effect that the efficiency of uptake may have on the efficacy of RNAi experiments. The experiments were based on the reasoning that uptake of dsRNA into cells may vary between insect species and this variability could be responsible for the variability in sensitivity to RNAi observed in insects (Terenius *et al.*, 2011). It has been established that some insect cells are deficient in dsRNA uptake when isolated and grown under controlled conditions (Beck & Strand, 2003, 2005; Johnson *et al.*, 2010). However, *in vivo* experiments, which provide more relevant information with regards to the success of *in vivo* RNAi experiments, have hitherto been lacking. This chapter presents data on the *in vivo* uptake of dsRNA in *Manduca sexta*, in which RNAi success has been at best variable and in some cases lacking. In addition, the presence in the *M. sexta* transcriptome of SID-1, a potentially important molecule for systemic RNAi, is reported.

In order to study the uptake of dsRNA into insect tissue *in vivo* three assays were developed. The first involved the detection of an injected non-insect dsRNA molecule (coding a section of eGFP sequence) with a modified q-RT-PCR protocol. Calibration of the assay using known quantities of dsRNA (Figure 5.1a) confirmed this assay as a viable technique for the detection of exogenous dsRNA. The assay was able to detect the presence of significant quantities of dsRNA in *M. sexta* fat body and midgut tissue and in hemocytes following dsRNA injection (Figure 5.1b). This experimental technique involved the detection of injected dsRNA with PCR, which is extremely sensitive. Caution must therefore be displayed in the interpretation of these data, since any contaminating dsRNA that had not been removed from the tissue during washing procedures could have resulted in a significant PCR signal and false positive results. An appropriate contamination control, which was not used in this study, but which should be included in future studies of this kind, would be to bathe untreated tissue in the dsRNA solution, wash the tissue as usual and analyse by q-RT-PCR.

The second method used to study uptake into tissues involved the detection of fluorescent dsRNA in insect hemocytes by flow cytometry. This detection method was also successful, with hemocytes from insects injected with fluorescently-labelled dsRNA showing elevated fluorescence levels compared to hemocytes from control insects (Figure 5.2). Moreover, the fluorescence of hemocytes was dependent on the dose of dsRNA injected. The final experimental technique also involved the detection of fluorescent dsRNA. In this assay *in vivo* uptake of dsRNA was analysed using confocal microscopy; fluorescent dsRNA was

injected into larvae and hemocytes were subsequently removed from the insect, allowed to form a monolayer on a coverslip, fixed, stained and visualised using confocal microscopy. Significant internalisation of dsRNA into hemocytes (predominantly plasmatocytes) was detected using this technique (Figure 5.5). An *in vitro* variant of this assay was also performed, where dsRNA was detected in hemocytes following incubation of a hemocyte monolayer in media containing fluorescent dsRNA (Figure 5.6).

The flow cytometry and confocal microscopy methods involved the detection of a fluorescent signal following the injection of fluorescent dsRNA. Prudence must be exercised in drawing conclusions from these experiments since the detected fluorescence signals are not necessarily in the form of dsRNA, but could instead represent fluorescent label released from the RNA by a breakdown process. Furthermore, regarding the confocal microscopy experiments, it is not possible to be sure that the fluorescent dsRNA was taken up into the cells *in vivo,* since dsRNA uptake could have occurred following dissection of hemolymph and incubation of hemocytes *in vitro.* However, the results are consistent with uptake *in vivo*; uptake during the *in vitro* stage of the experiments was unlikely to have taken place because little uptake was observed in hemocytes from treatments where the time between dsRNA injection and dissection (*in vivo* incubation time) was very short (Figure 5.5b), despite the fact that the incubation time after bleeding was the same in all treatments. Furthermore, uptake into hemocyte in the *in vitro* stages of the experiment was unlikely because the hemocytes were immediately washed and resuspended following bleeding, thereby removing any unincorporated labeled dsRNA. The most appropriate interpretation of this data is, therefore, that uptake of dsRNA into hemocytes occurred *in vivo*.

Despite some uncertainty surrounding the interpretation of the uptake experiments, taken together, the results obtained from three separate assays indicate that uptake of dsRNA (at time points relevant to RNAi experiments) is not deficient in *Manduca sexta*. It is, therefore, not likely that insufficient uptake of dsRNA is responsible for the variable efficacy of RNAi in this insect.

5.3.2 Is there greater uptake into particular tissues?

In addition to asking whether uptake of injected dsRNA is sufficient for *in vivo* RNAi in *M. sexta* it is also of interest to ask whether there are differences in the efficiency of uptake in different tissues, since differences in uptake of dsRNA could be responsible for the variable susceptibility of different tissues to RNAi. There is some evidence for variability in sensitivity to RNAi in insect tissue. Terenius *et al.* (2011) reported that hemocytes, fat body,

midgut and brain tissue were in general rather susceptible to RNAi, whereas epidermal tissue (larval epidermis and pupal wing) were rather refractory. The q-RT-PCR experiments yielded data regarding uptake into three different tissues in *M. sexta*. There appeared to be more uptake into fat body than midgut tissue (Figure 5.1b). Fat body tissue may be to be exposed to greater quantities of dsRNA circulating in the hemolymph, or may simply be better able to take up dsRNA.

The q-RT-PCR data also indicate that there appeared to be more uptake of dsRNA into hemocytes than into fat body and midgut tissue, a finding consistent with the finding of Miller *et al.* (2008) that hemocytes had a superior ability (compared to several other tissues tested) to take up dsRNA in *D. melanogaster* larvae but not in accord with my observation that expression of the SID-1-like gene in haemocytes is less than in midgut or fat body. However, it must be admitted that it is difficult to compare the relative quantities of dsRNA taken up into hemocytes (compared with other tissues), because the mass of haemocyte tissue in hemolymph was not measured (there are good reasons why this is difficult, including the rapid coagulation of hemolymph, and the difficulty of washing haemocytes without losing them). As a consequence, different units were employed to express the levels of dsRNA (dsRNA was quantified per microlitre of hemolymph for hemocytes and per microgram of tissue for midgut and fat body tissue). Therefore, any conclusion drawn from this comparison should be regarded as preliminary. In order to be able to make a more meaningful evaluation it would be necessary to express the quantity of dsRNA per cell number or cell mass; these quantities could be estimated by measuring the DNA or protein content of the washed haemocyte pellet.

Miller *et al.* (2008) did not propose an explanation for the apparently superior ability of *Drosophila melanogaster* hemocytes to internalise dsRNA. Insect hemocytes represent an important component of the insect immune system and perform various defence roles including phagocytosis, nodulation and encapsulation (Lavine & Strand, 2002). They are also known to remove large particulate matter (Wigglesworth, 1972) and macromolecules (Das *et al.*, 2008) from the hemolymph. I therefore propose that hemocytes, as part of their role in immunity, may internalise dsRNA in order to protect other tissues from foreign dsRNA molecules and in order to mount an effective immune response against any potential infecting agent.

The flow cytometry experiments clearly indicated that a subset of hemocytes more readily takes up dsRNA than the hemocyte population in general. Analysis of the properties of the hemocytes appearing to take up the greatest quantities of dsRNA revealed they are likely to be small in size and low in granularity. These observations enable a hypothesis to be formed

as to the subpopulation of hemocytes responsible for the majority of dsRNA uptake. For instance, small plasmatocytes could be proposed as responsible, since in most lepidopteran species cells with this description are devoid of granules (Raina, 1976; Essawy *et al.*, 1985; Butt & Shields, 1996; Ribeiro *et al.*, 1996) and are therefore likely to display low side scatter values during analysis by flow cytometry.

In order to ascertain whether distinct hemocyte populations were responsible for the majority of dsRNA uptake confocal microscopy analysis was employed. The *in vivo* experiments found that the majority of fluorescent dsRNA was taken up into plasmatocytes (Figure 5.5), whilst an *in vitro* assay found that granular cells were responsible for the majority of dsRNA uptake (Figure 5.6). There are several potential explanations for these contrasting results; the first being that the ability of plasmatocytes to take up exogenous dsRNA might have been compromised in the *in vitro* assay by the relatively reduced area of plasmatocyte plasma membrane that was exposed to the bathing medium once they had settled and attached to the glass surface (since plasmatocytes spread extensively on glass while granular cells do not). An alternative explanation is that spreading induced other cellular changes (for example, a reduced ability to internalise and recycle membrane-bound vesicles through the endocytic pathway) which reduced the ability of the plasmatocytes to take up dsRNA *in vitro*. Conversely *in vitro* incubation may have had some positive effect on granular cells that enhanced their ability to take up dsRNA. Activation of granular cells (to be more readily able to internalise dsRNA) could have occurred as a result of the experimental bleeding process, which may have induced the production of cytokines with the ability to activate relevant signalling cascades. It has been shown that expression of cytokines can be induced by tissue damage in insects (Pastor-Pareja *et al.*, 2008), so this hypothesis is not outside the realm of possibility.

The *in vitro* observations may not, after all, be the result of preferential uptake into granular cells, but rather, result from the differential uptake dynamics *in vitro* and *in vivo*. We know that there is enzymatic breakdown of the exogenous dsRNA *in vivo* (see Chapter 4, Figures 4.1 & 4.2a), and this is likely to compete with uptake of dsRNA *in vivo*, but will either not occur, or will occur at a lower rate *in vitro* because the hemolymph plasma was removed during the preparation of the haemocyte monolayer. Therefore, plasmatocytes may have taken up dsRNA preferentially *in vitro* (as seen *in vivo*) but may have only a limited and saturable capacity to do so. Because exogenous dsRNA was probably more available for uptake in the *in vitro* experiments (due to the lack of competing enzymatic breakdown), more dsRNA would be available to granular cells, and the degree of uptake observed *in vitro* may simply be a result of this. In any case, since there are many factors that could influence the outcome of the *in vitro* experiment, and since the *in vivo* data can be considered to be

more biologically relevant, I place more significance on the *in vivo* data and conclude that dsRNA is preferentially taken up into plasmatocytes in *M. sexta* larvae.

Additional methods could be used to study the identity of the cell population responsible for the majority of dsRNA uptake into *M. sexta* hemocyte. Further flow cytometry, or "cell-sorting", experiments whereby cells that displayed high fluorescence were sorted from the main population could be useful, since analysis of the sorted cells by microscopy could expose their identity. Alternatively, the use of hemocyte-specific antibodies (as used by Zhuang *et al.*, 2008) could aid in the identification of any hemocyte populations preferentially taking up dsRNA.

There are no published studies, to my knowledge, concerned with the differential uptake of dsRNA into incongruent hemocytes populations. In the case of the phagocytic role of hemocytes there is some evidence for disparate roles for different classes of hemocytes. Costa *et al.* (2005) observed that, in *Spodoptera littoralis*, granular haemocytes are the primary phagocytic haemocytes. Ling & Yu (2006) found that *M. sexta* plasmatocytes were the major hemocytes involved in phagocytosis of foreign (non-self) particles, whereas granulocytes were the only hemocytes to phagocytose dead (self) cells. The results presented here indicate that, in *Manduca sexta*, plasmatocytes have a major role in the sequestration of dsRNA molecules, but that granular cells are also competent to take up dsRNA. The observation that a small subset of hemocytes appear to be taking up dsRNA raises the question of whether there is variability in susceptibility to RNAi among hemocyte populations, as there is clearly variability in dsRNA uptake. There is no evidence for variability in efficacy of RNAi in different hemocyte populations. In fact it seems that several different hemocyte populations are susceptible to RNAi. For example, Zhuang *et al.* (2008) were able to use *in vivo* RNAi to effectively knock down three integrin alpha (α) subunits (α1, α2 and α3), which are expressed in different populations of hemocytes (small plasmatocytes, granular cells and some large plasmatocytes and granular cells respectively).

In light of the conclusions drawn so far concerning different cell populations, the possibility must be considered that not all of the hemocytes circulating in *M. sexta* larval hemolymph were analysed during the flow cytometry and confocal microscopy experiments, since not all hemocytes would have been removed from the insect when the hemolymph was dissected (some cells are likely to have remained attached to internal tissues). Therefore, it is possible that our analysis of uptake into hemocyte populations is not complete, since data would be lacking on the uptake of dsRNA into those cells which were not effectively removed. Indeed, only plasmatocytes and granular hemocytes were identified in the monolayer preparations. Additional experiments concerned with removing from larvae any attached

hemocytes, perhaps by injecting anticoagulant saline, would be necessary to ensure that all hemocytes were analysed.

5.3.3 What was the mode of action of uptake?

There has been some interest concerning the cellular mechanism of uptake at work during internalisation of dsRNA in insect cells. In a screen to identify genes required for RNAi in *Drosophila melanogaster* S2 cells Ulvila *et al.* (2006) identified components of the endocytic machinery (including clathrin heavy chain and two scavenger receptors, SR-C1 and Eater). They also observed that blocking endocytosis in S2 cells impaired RNAi, implicating receptor-mediated endocytosis (an active process) in the internalisation of dsRNA. Saleh *et al.* (2006) also found that dsRNA is taken up by an active process involving receptor-mediated endocytosis n *Drosophila melanogaster* S2 cells and *Caenorhabditis elegans* worms. The confocal microscopy data presented here contrast with these findings, since uptake was found to be able to occur at reduced temperatures (Figures 5.5e & 5.6e), indicating that uptake of dsRNA was taking place via a passive process and did not rely on active cellular processes. Introduced dsRNA did, however, appear to be located in structures in the perinuclear region, likely to be vesicles of some kind, which is consistent with uptake via endocytosis, but may also signify concentration of dsRNA into vesicles following uptake by another method.

The results of the *in vivo* experiments conducted using *M. sexta* are not entirely consistent with work undertaken in cultured *Drosophila melanogaster* cells. This disparity in results could have arisen from differences in uptake methods amongst insect taxa, dsRNA uptake may occur by receptor-mediated endocytosis in *D. melanogaster,* but use a different method in lepidopteran insects. This seems a rather unlikely explanation. It is perhaps more reasonable to think that the observed difference in uptake are the result of the different behaviour of cultured cells compared to cells *in situ.*

5.3.4 Is SID-1 expressed in Manduca sexta *tissue?*

A different approach to studying the cellular internalisation of introduced dsRNA was to determine whether *M. sexta* encodes certain proteins known to be important for systemic RNAi. In the nematode worm, *C. elegans,* the SID-1 (systemic RNA interference defective) protein is required to allow the spread of RNAi interference information between tissues, leading to a systemic RNAi response (Winston *et al.*, 2002). Lack of a *sid-1* gene has been

linked to insufficient systemic RNAi in *Drosophila melanogaster* (Gura, 2000; Roignant *et al.*, 2003) and it has been suggested that insects that encode *sid-1*-like genes are sensitive to systemic RNAi, whilst those that do not encode *sid-1*-like genes are defective in environmental RNAi (Tomoyasu *et al.*, 2008). This study reports the presence of a *sid-1*-like gene in *Manduca sexta*, which is expressed at reasonable levels in larval tissue, indicating that a lack of *sid-1*-like gene products is unlikely to be responsible for the observed variable response to RNAi in *M. sexta.*

Whilst the data presented here indicate that the lack of a *sid-1*-like gene in the *M. sexta* genome or the deficiency in the expression of a *sid-1*-like gene is not likely to be responsible for the variable sensitivity of this insect to RNAi, it is still not entirely clear what role SID-1 proteins play in insect RNAi. The story is further complicated because insects are known to encode variable numbers of *sid-1*-like genes. *Tribolium* and *Bombyx*¸ for instance, each encode three *sid-1*-like genes (Tomoyasu *et al.*, 2008), whilst the honeybee genome harbours only a single *sid-1* homologue (Honeybee Genome Sequencing Consortium, 2006). The discovery that *Manduca sexta* encodes one *sid-1* homologue, which has high inferred amino acid identity to the *Bombyx mori* SID-1-related 3 protein, cannot confirm that this insect should be susceptible to systemic RNAi, since without full genomic information we cannot know how many *sid-1-like* genes are present in this insect. In any case, we do not yet understand the function of the many *sid-1-like* genes identified in insects. In order for conclusions of this nature to be drawn there needs to be a better understanding of the functioning and redundancy of *sid-1*-like gene products with respect to systemic RNAi. Reverse genetic experiments that selectively removed the function(s) of individual *sid-1-like* genes using an insect that reproducibly displays good RNAi knockdowns (i.e. *Tribolium castaneum*) would probably be a good experimental approach.

5.4 Methods

5.4.1 q-RT-PCR assay

In order to quantitate uptake of dsRNA into tissue an assay was developed whereby introduced dsRNA with the GFP sequence was detected using q-RT-PCR. The assay was calibrated by spiking dissected cell-free hemolymph with a range of doses of dsRNA and analysing the spiked hemolymph by q-RT-PCR with eGFP primers (eGFP_qPCR_F: 5'-CCG ACC ACT ACC AGC AGA AC-3'; eGFP_qPCR_R: 5'-TTG GGG TCT TTG CTC AGG-3'). Experiments could then be conducted by which injected dsRNA could be detected

in tissue using q-RT-PCR and quantified using the equation derived from the calibration data.

In the first experiment to detect dsRNA uptake 4 µg dsRNA was injected into newly emerged fifth instar *M. sexta* larvae (injections were conducted as described in section 2.2). Tissue was dissected 6 hours post injection (see section 2.2), and a note taken of the weight of the tissue. Total RNA was extracted from the tissue, treated with DNase and an aliquot reverse transcribed, as described in section 2.10, except that the amount of RNA used in the reverse transcription reaction was adjusted for all samples to be equivalent to a tissue weight (for example, RNA extracted from approximately 20 mg of tissue was used in the reaction). q-RT-PCR was performed on a dilution of the resulting cDNA as previously described. Ct values obtained from the qPCR reactions were used to calculate the quantity of dsRNA in the tissue, using the formula derived from the calibration experiment.

5.4.2 Flow cytometry experiments

Fluorescent dsRNA was synthesised by *in vitro* transcription as previously described (section 2.11) except that 50% of the dUTP in the reaction was replaced with fluorescein-12 labelled dUTP (Yorkshire Bioscience). The DNA template sequence used to synthesise the dsRNA was eGFP: a sequence presumed not to be present in the *M. sexta* genome was chosen because it was beneficial not to carry the risk of knocking down any genes and therefore potentially altering the processing of dsRNA. Fluorescence of the dsRNA was confirmed by running a sample (alongside a non-labelled dsRNA as a control) on an agarose gel that did not contain ethidium bromide. Visualisation of the dsRNA with a transilluminator confirmed that the dsRNA was fluorescently labelled.

All samples studied were analysed on a BD FACSCanto™ flow cytometer. Both forward scatter (FSC-H) and side scatter (SSC-A) voltages were adjusted to appropriate values to allow analysis. Events smaller than 25,000 units on both scales were not counted, as these were likely to result from dust particles. Cells were analysed for fluorescein 12 (530 ± 30 nm) using a 488 nm laser. Thirty thousand events were recorded for each sample. Results were analysed using BD FACSDiva™ software.

In the first FACS experiment insects were injected with 50 µl DEPC-treated water containing 1 µg, 5 µg or 10 µg fluroscein-12 labelled dsRNA for GFP, or with a control injection of 50 µl DEPC-treated water (injections were conducted as described in Section). Treated insects were incubated at 25°C for one hour, after which they were dissected for

hemolymph (see Section 2.2). Hemolymph (approximately 100 µl) was collected into individual prechilled polypropylene 1.5 ml microcentrifuge tubes (Sarstedt) containing 900 µl Manduca buffered saline solution (MBS; 4 mM NaCl, 40 mM KCl, 18 mM $MgCl_2 \cdot 6H20$, 1.5 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 192.8 mM sucrose, pH 4.5). To separate hemocytes from the hemolymph plasma the samples were centrifuged at 1,000 x g for 8 minutes at 4°C, and the supernatant discarded. Pelleted hemocytes were re-suspended in 1 mL Grace's Insect Medium (GIM) (Sigma), centrifuged again as above, the supernatant removed and the cells again re-suspended in 1 ml GIM. All work was carried out on ice.

The time-course experiment was conducted as described above, except that all dsRNA-treated insects were injected with 5 µg dsRNA. The incubation time between fluroscein-12 labelled dsRNA injection and dissection of the hemolymph was either 15 minutes, 30 minutes, 1 hour or 2 hours.

5.4.3 Confocal microscopy

In the *in vivo* dsRNA internalization assay newly emerged fifth instar larvae were injected with 50 µl DEPC-treated water or 50 µl DEPC-treated water containing 10 µg fluorescein-12-labelled dsRNA (synthesized as describe above). Following incubation of the injected larvae at room temperature for a number of hours hemolymph was removed from the treated insects by bleeding into a prechilled microcentrifuge tube on ice (as described in detail in section 2.2). In order to facilitate the separation by centrifugation of hemocytes from hemolymph plasma, hemolymph from two insects was bled into a single microcentrifuge tube. The hemolymph samples were centrifuged at 250 x g for 5 minutes at 4°C, after which the walls of the microcentrifuge tubes were examined for the presence of a hemocyte pellet. If the pellet was not visible the sample was centrifuged under the same conditions for a further 3 minutes. Once a visible pellet had formed the supernatant was carefully removed from the tubes by pipetting, 500 µl of ice cold Grace's Insect Media (GIM; Sigma) was added to the hemocyte pellets and the samples were again centrifuged at 250 x g for 5 minutes at 4°C. The supernatant was carefully removed from the tubes and the hemocytes resuspended by pipetting in 500 µl ice cold GIM. 10 mm coverslips (0.17 mm thick) were prepared by soaking in 70% ethanol and rinsing in sterile distilled water and placed centrally in wells of a 24 well plate (Sarstedt). The hemocytes suspension (500 µl) was pipetted onto each coverslip and left undisturbed for 30 min (at room temperature in the dark) to allow the hemocytes to settle and form a monolayer.

Subsequent to the formation of a hemocyte monolayer the supernatant was removed from the wells, after which the monolayers were washed once with 500 µl room temperature GIM

and fixed in 4% (w/v) paraformaldehyde (PFA) in PBS. 500 µl PFA was added to each well, working in a fume hood, and the plate was incubated at at 4°C for 1 hour. The cells were then washed three times in PBS, permeabilised in 0.1% (v/v) Triton X-l00 (Sigma) for 5 minutes, washed three times in PBS and non-specific binding sites were blocked with 0.5% (w/v) bovine serum albumin (BSA; Sigma) in PBS for 30 minutes. Hemocytes were then stained with phalloidin conjugated to fluorescein isothiocyanate (FITC–phalloidin; Sigma); 250 µl of FITC–phalloidin solution (1 $\mu g ml^{-1}$, final concentration in PBS) was added to each well, cells were stained at room temperature for 30 minutes in the dark and then washed three times in PBS and once in distilled water. Monolayers were mounted overnight at room temperature in 5 µl Mowiol-4-88 solution (Calbiochem, Nottingham, UK) and stored at 4°C. All cell preparations were visualized using a Zeiss Confocal Laser Scanning Microscope (LSM510META).

The *in vitro* dsRNA internalization assay was conducted as above except that hemolymph was collected from untreated insects. Separated hemocytes were resuspended in 1 ml GIM and 500 µl of this cell suspension was added to coverslips in wells of a 24 well plate. After the hemocytes had settled and formed monolayers 250 µl ice cold GIM or 250 µl ice cold GIM containing 5 µg/ml fluorescein-12-labelled dsRNA was pipetted into the wells and the plates incubated at room temperature. The supernatant was carefully removed by pipetting, the cells washed once with room temperature GIM and fixed and stained as described above.

5.4.4 Molecular cloning of Manduca sexta *SID-1*

A fragment of a *M. sexta sid-1* gene was cloned by RT-PCR using degenerate primers based on the conserved amino acid sequences FNHVFSN (forward primer) and NFQFDT (reverse primers) identified by Tian *et al* (2009). The sequence of the primers was: SID1F1 5'-TTY AAY CAY GTN TTY WSN AA-3' and SID1R1 5'-NTG RTC RAA YTG RAA RTT-3'. In order to potentially enrich for *sid-1* mRNA fat body tissue was dissected from a newly emerged fifth instar larva insect 6 hours after injection with dsRNA. Extracted RNA was treated with RNase-free DNaseI (Ambion) and reverse transcribed using MMLV reverse transcriptase (Promega) with random hexadeoxynucleotide primers. PCR reactions were performed using Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen), standard reagents (section 2.6) and the conditions employed were heating to 95°C for 10 minutes, followed by 50 cycles of 95°C for 40 seconds, 40°C for 30 seconds and 68°C for 1 minute and a final extension of 68°C for 10 minutes. A second round of PCR was performed using the same primers and a dilution of the product from the first round (1/250) as a template,

yielding a PCR product of the appropriate size, which was cloned and sequenced as described in section 2.8.

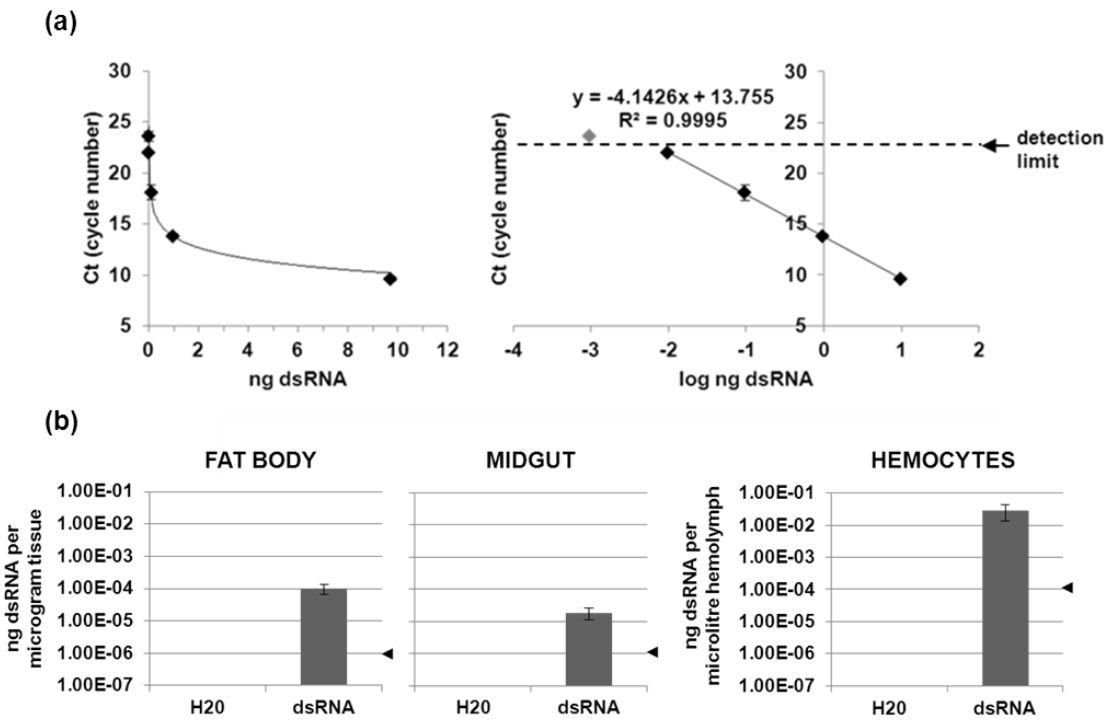


Figure 5.1: Uptake of dsRNA into *Manduca sexta* tissue as detected by q-RT-PCR. (a) The assay was calibrated by spiking dissected plasma with a range of doses of dsRNA for eGFP and by carrying out q-RT-PCR. One data point (in grey) was excluded from the fitted trendline because it was interpreted as falling below the detection limit of the assay. The equation of the fitted line enabled the computation of dsRNA quantity for a given qPCR output. **(b)** Levels of dsRNA detected in *Manduca sexta* tissue following injection with dsRNA (dsRNA) or with water (H_20) (n=3). Newly ecdysed 5^{th} instar larvae were injected with either 4 µg dsRNA or DEPC-treated water and dissected for tissue 6 hours later. dsRNA levels were quantified in tissue using q-RT-PCR. Data are the mean ± standard error. The detection limit of the assay is marked with a black arrow.

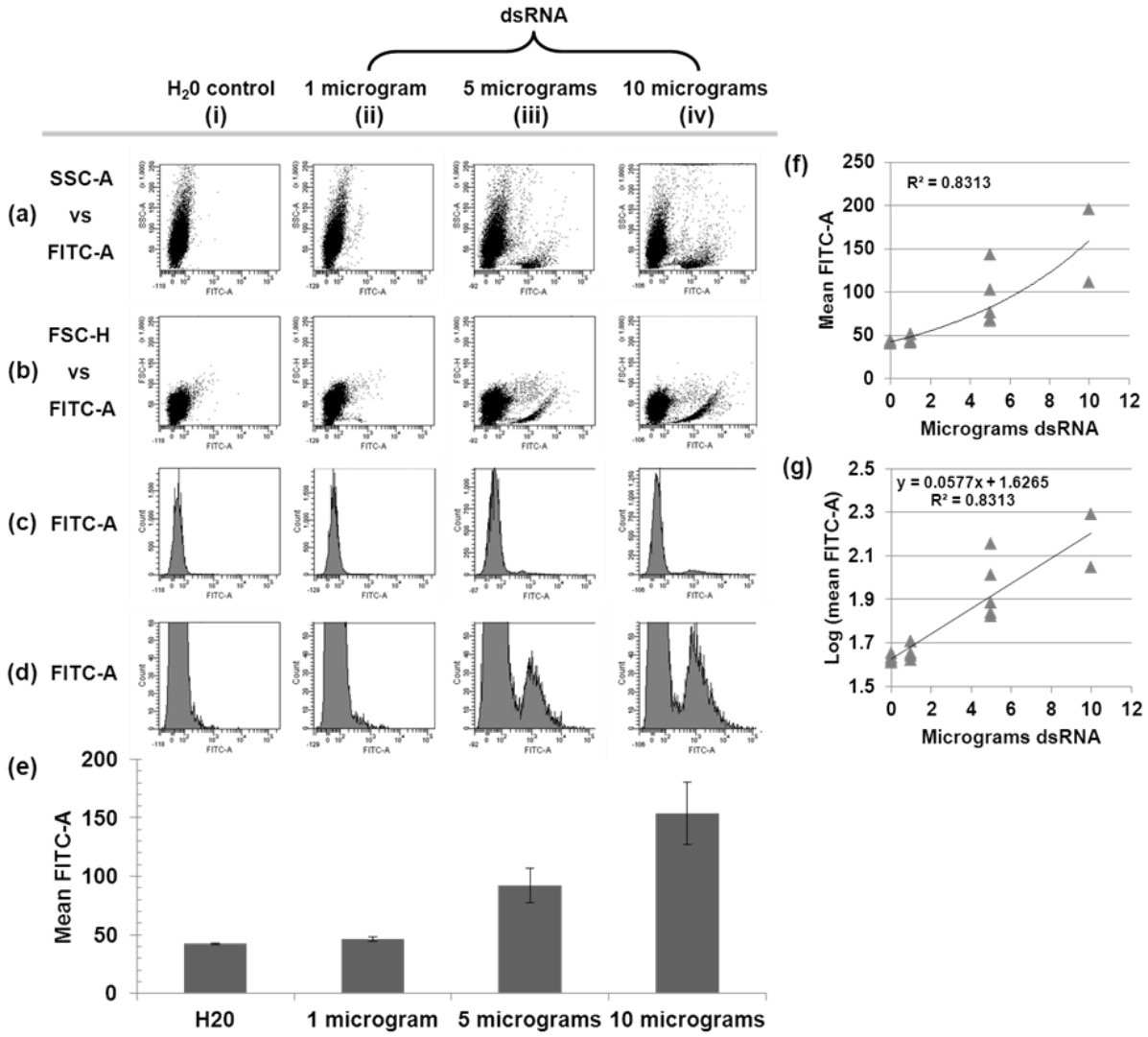


Figure 5.2: Uptake of dsRNA into hemocytes *in vivo*. Newly emerged 5th instar *Manduca sexta* larvae were injected with either (i) DEPC-treated water (H20 control) or (ii-iv) different doses of fluoroscein-12-labelled dsRNA. One hour post injection hemocytes were dissected and subjected to flow cytometry analysis. **(a)** is a scatter diagram displaying 30,000 events with the side scatter of the light (SSC-A) on the y-axis and the levels of fluoroscein-12 detected (FITC-A) on the x-axis. **(b)** is a scatter diagram displaying 30,000 events with the forward scatter of the light (FSC-H) on the y-axis and the levels of fluoroscein-12 detected (FITC-A) on the x-axis. **(c)** is a histogram of FITC-A and **(d)** is a histogram of FITC-A with a reduced scale on the y-axis. Results displayed in (a)-(d) are one of five replicates (for the H20, 1 microgram and 5 microgram injection treatments) and one of two replicates (for the 10 microgram injection treatment). All other replicates are included in the Appendix Figure A1. **(e)** Mean fluorescence of the 30,000 cells analysed per sample was taken as a single data point and the bar chart shows the mean of this mean fluorescence value for 5 replicates (for the H20, 1 microgram and 5 microgram injection treatments) and for two replicates (for the 10 microgram injection treatment). The data therefore represents the mean of the mean fluorescence levels (FITC-A) of the cells ± standard error. **(f)** Scatter diagram of mean fluorescence (FITC-A) versus dose dsRNA with an exponential trend line fitted. **(g)** Scatter diagram of log transformed FITC-A versus dose dsRNA with a linear trend line fitted.

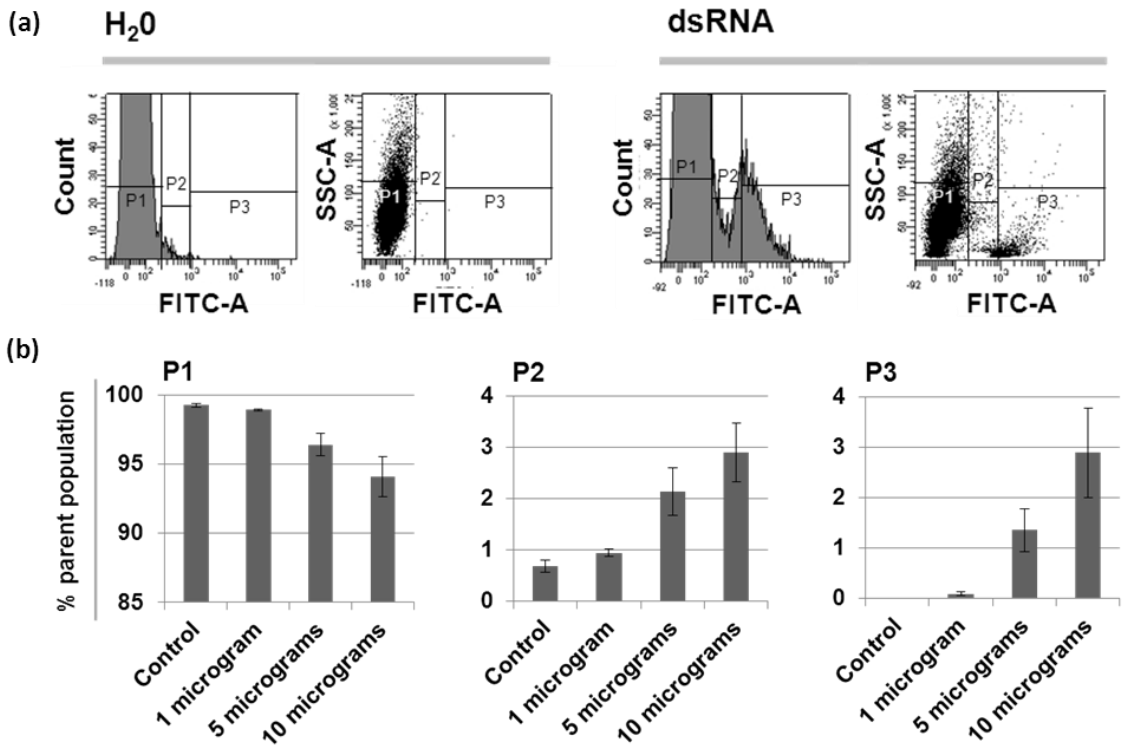


Figure 5.3: A small proportion of hemocytes have increased fluorescence following injection with fluorescent dsRNA. Cells from the flow cytometry experiment were gated into three different populations based on their fluorescence (FITC-A). Population 1 (P1) is composed of cells with low fluorescence and includes 99% of cells in H_20-injected insects. Populations 2&3 (P2&P3) are composed of cells with medium and high fluorescence respectively. **(a)** Population boundaries displayed on the histogram of fluorescence and the scatter diagram of fluorescence versus side-scatter for cells from insects injected with water (H_20) and for cells from insects injected with 5 micrograms dsRNA (dsRNA). **(b)** Mean % of cells falling into the three populations ± standard error in cells originating from the different injection treatments.

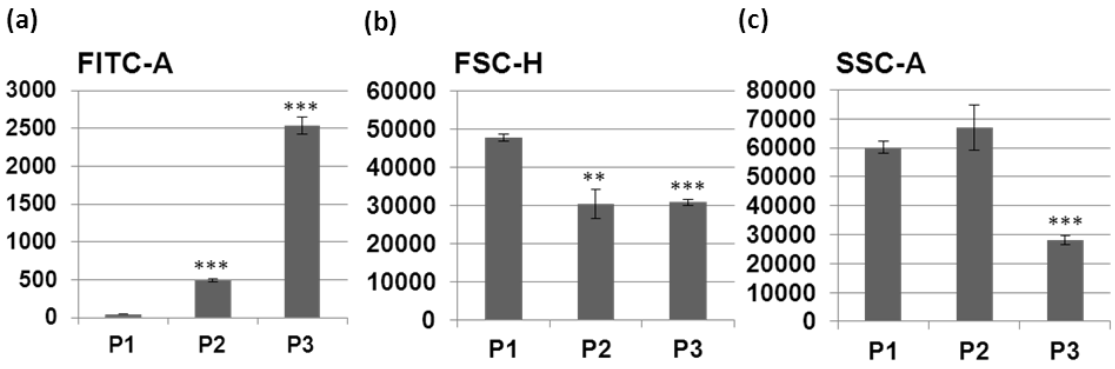


Figure 5.4: Hemocytes with increased fluorescence have relatively low forward scatter and side scatter values. Hemocytes derived from insects injected with 5 micrograms dsRNA (n=5) were gated according to their fluorescence. Population 1 (P1) is composed of cells with low fluorescence and includes 99% of cells in H_20-injected insects. Populations 2&3 (P2&P3) are composed of cells with medium and high fluorescence respectively. **(a)** Mean fluorescence (FITC), **(b)** mean forward scatter (FSC-H) and **(c)** mean side scatter (SSC-A) ± standard error of the gated cell populations. Significance differences in FITC-A, FSC-H and SSC-A values from population 1 are denoted using the following terminology for the p-values obtained from the paired t-tests performed on the transformed data: 0.1 (.), 0.05 (*), 0.01 (**), and 0.001 (***).

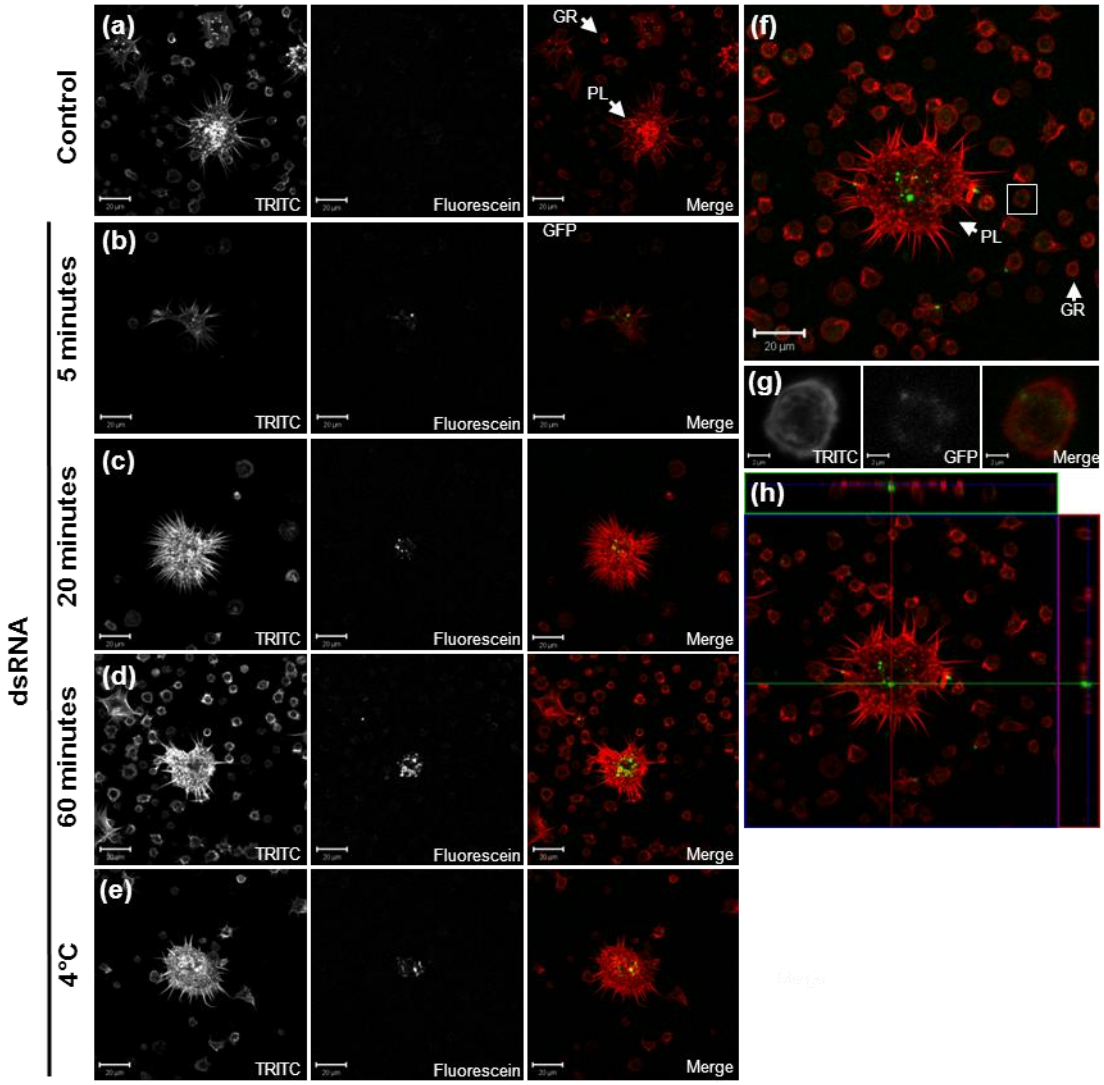


Figure 5.5: dsRNA is internalised in *M. sexta* hemocytes *in vivo*. DEPC-treated water (control) or DEPC-treated water containing 10 µg fluorescein-labeled dsRNA (dsRNA; green in merge) was injected into newly emerged fifth instar larvae, from which hemocytes were dissected, adhered to coverslips, fixed and stained with TRITC-linked phalloidin (red in merge). **(a)-(e)** Single 2 µm confocal sections of hemocytes dissected from larvae 60 minutes post injection with DEPC-treated water (a) or hemocytes dissected from larvae a number of minutes post injection with fluorescein-labeled dsRNA (b)-(d). Hemocytes in (e) were collected from larvae 60 minutes post injection with fluorescein-labeled dsRNA, when the insects had been incubated at 4°C. **(f)** 3D projection from zstack of hemocytes collected from larvae 60 minutes after injection with fluorescein-labeled dsRNA. **(g)** Zoom of granular cell from (f) **(h)** Single 2 µm confocal section with orthogonal projections derived from zstack shown in (f). Scale bars are 20 µm (except in (g) where they are 2µm). Plasmatocytes (PL) and granular cells (GR) are labeled with white arrows.

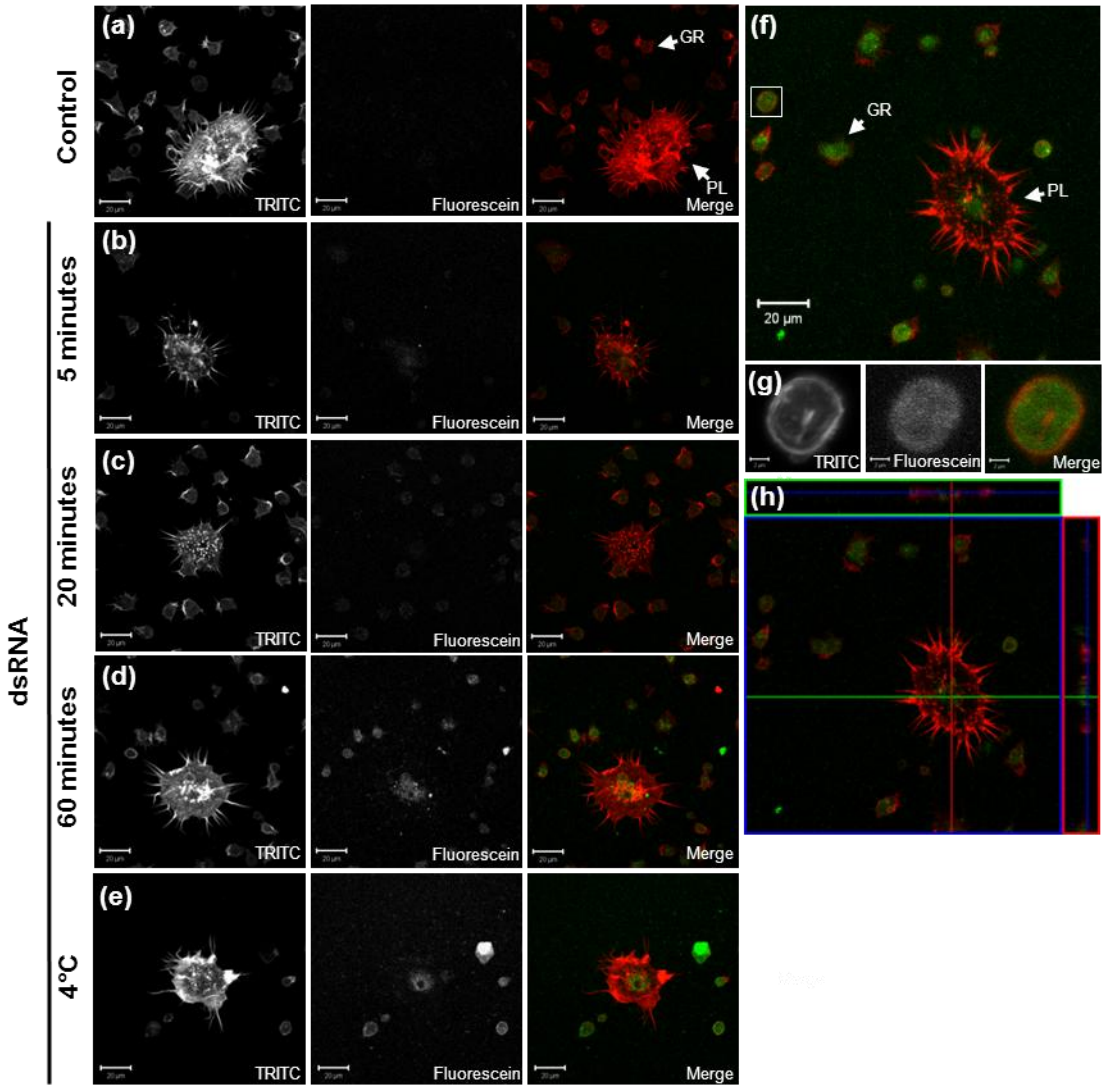


Figure 5.6: dsRNA is internalised in *M. sexta* hemocytes *in vitro*. Hemocytes were dissected from newly emerged fifth instar larvae, adhered to coverslips, incubated with GIM (control) or GIM containing 5 µg/ml fluorescein-labeled dsRNA (dsRNA; green in merge), fixed and stained with TRITC-linked phalloidin (red in merge). **(a)-(e)** Single 3 µm confocal sections of hemocytes incubated with GIM for 60 minutes (a) or GIM containing fluorescein-labeled dsRNA for a number of minutes at room temperature (b)-(d). Hemocytes in (e) were incubated with fluorescein-labeled dsRNA for 60 minutes at 4°C. **(f)** 3D projection from zstack of hemocytes incubated with fluorescein-labeled dsRNA for 60 minutes. **(g)** Zoom of granular cell from (f) **(h)** Single 2 µm confocal section with orthogonal projections derived from zstack shown in (f). Scale bars are 20 µm (except in (g) where they are 2µm). Plasmatocytes (PL) and granular cells (GR) are labeled with white arrows.

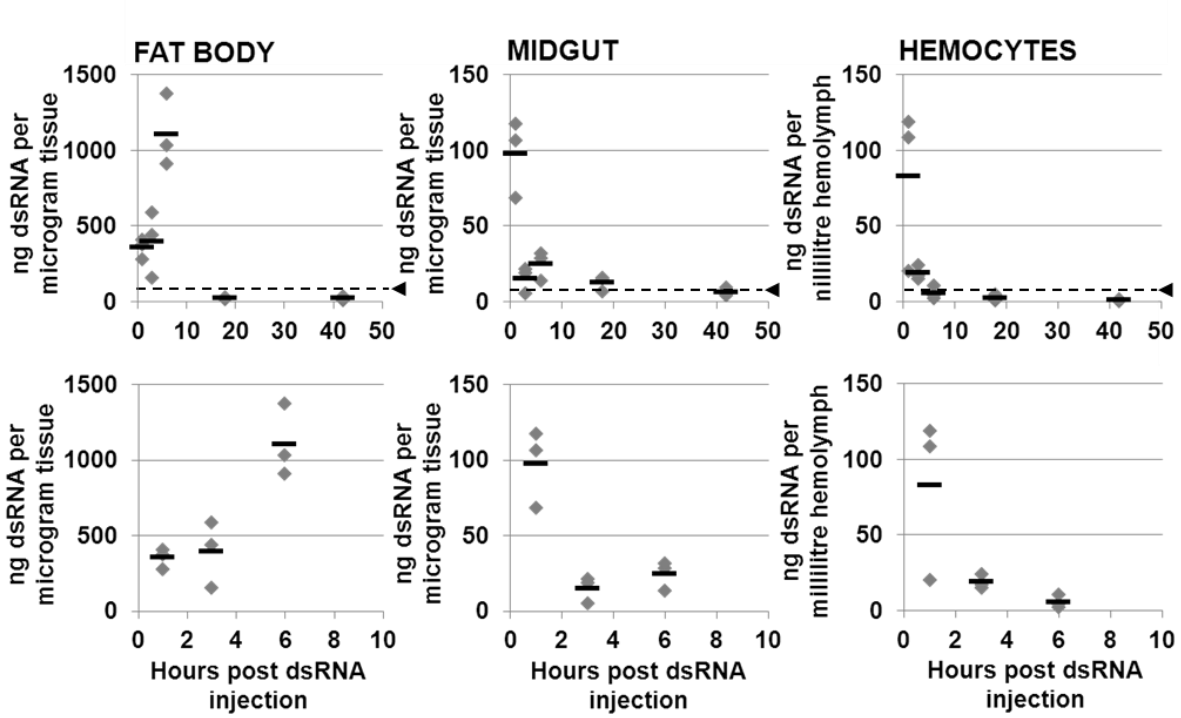


Figure 5.7: Time course of uptake into *Manduca* tissue (qPCR technique). Levels of dsRNA detected in *Manduca sexta* tissue following injection with dsRNA (n=3). Newly ecdysed 5th instar larvae were injected with 4µg dsRNA for eGFP and dissected for hemolymph, hemocytes, midgut and fat body a number of hours later. eGFP dsRNA levels were quantified in tissue using q-RT-PCR. The graphs depict data points (grey diamonds) and the mean (black dashes).

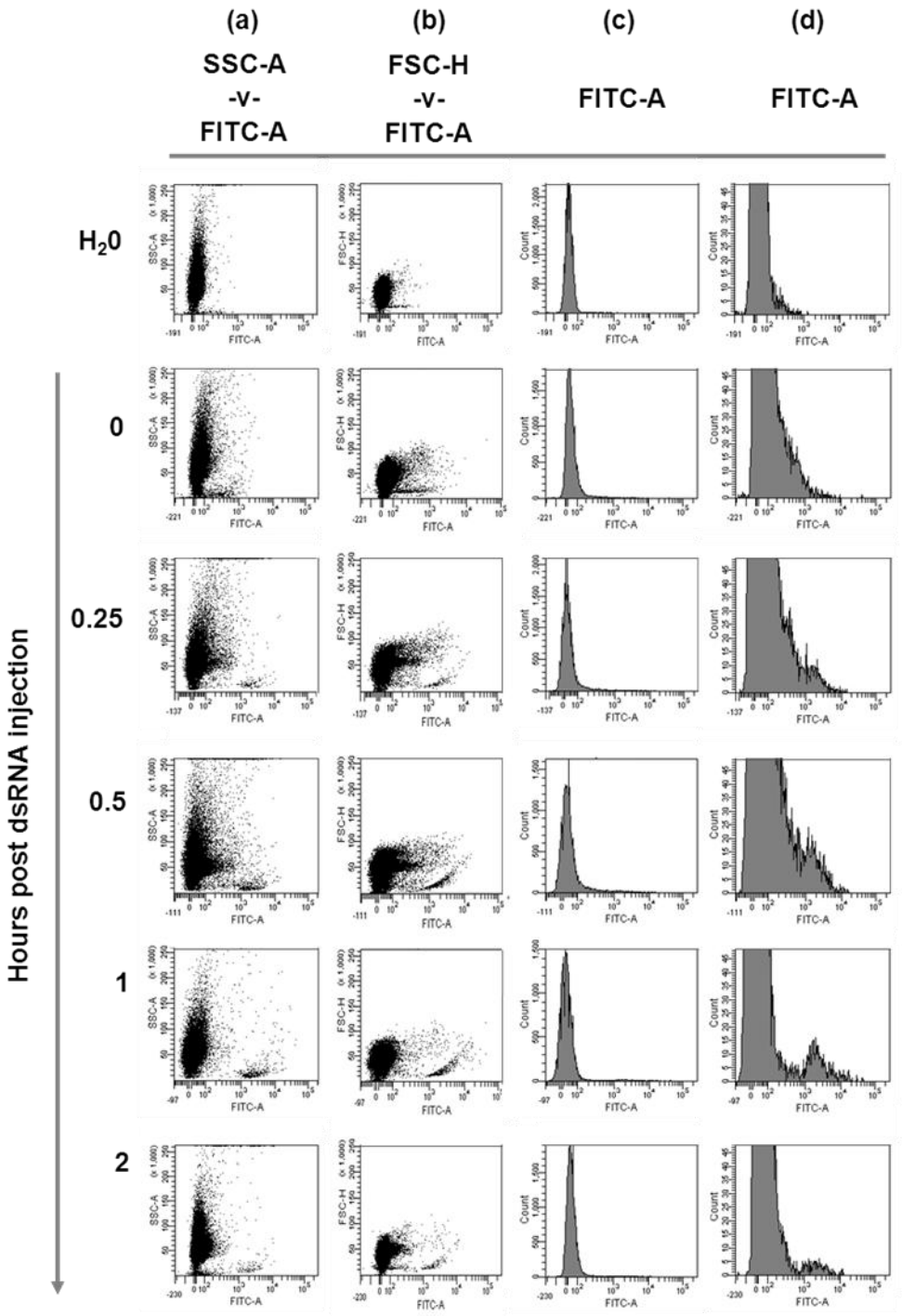


Figure 5.8: Time course of dsRNA uptake into cells (flow cytometry technique). Newly emerged *Manduca sexta* 5th instar larvae were injected with fluoroscein-12-labelled dsRNA and at varying intervals after injection hemocytes were dissected and subjected to flow cytometry analysis (n=2). **(a)** is a scatter diagram displaying 30,000 events with the side scatter of the light (SSC-A) on the y-axis and the levels of fluoroscein-12 detected (FITC-A) on the x-axis. **(b)** is a scatter diagram displaying 30,000 events with the forward scatter of the light (FSC-H) on the y-axis and the levels of fluoroscein-12 detected (FITC-A) on the x-axis. **(c)** is a histogram of FITC-A and **(d)** is a histogram of FITC-A with a reduced scale on the y-axis. Results displayed in **(a)-(d)** are one of two replicates. All replicates are included in the Appendix Figure A2.

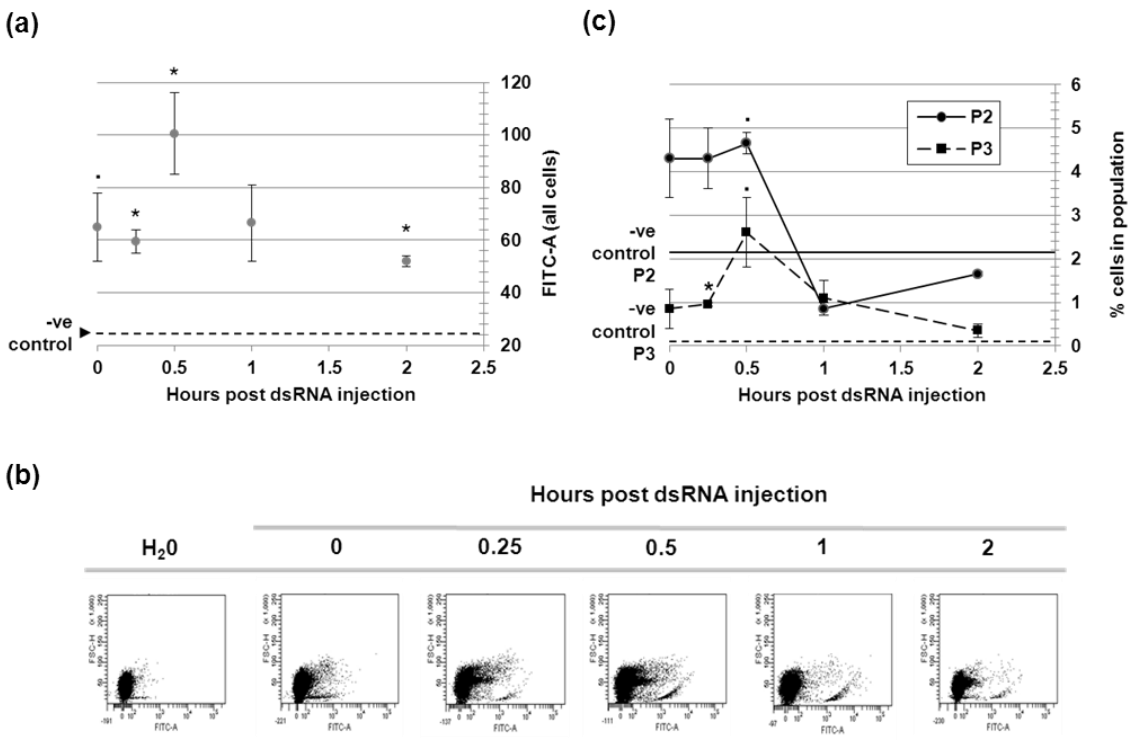


Figure 5.9: Time course of dsRNA uptake into cells (flow cytometry technique). Newly emerged *Manduca sexta* 5th instar larvae were injected with fluoroscein-12-labelled dsRNA and at varying intervals after injection hemocytes were dissected and subjected to flow cytometry analysis (n=2). **(a)** Mean fluorescence levels (FITC-A) in the cells ± standard error. **(b)** Fluorescence histograms displaying the population boundaries following the gating of cells into three populations (cells were gated into three different populations based on their fluorescence (FITC-A) - population 1 (P1) is composed of cells with low fluorescence and populations 2&3 (P2&P3) are composed of cells with medium and high fluorescence respectively). **(c)** mean % cells in gated populations ± standard error. Significantly increased cell numbers compared to the control (water-injection) are denoted using the following terminology for the p-values obtained from the t-tests performed: 0.1 (.) 0.05 (*), 0.01 (**), and 0.001 (***).

(a)

```
5′- GCG CTG TTC ATG CTG CAG CTG CGC CGC CGC CAG TGG AAC CGC AAG
CGG GCG CCG CGC GAC GAG GAG TAC GGC ATC CCG GCG CAC TAC GGA CTG
CTA TCG TCG CTA GGC GCG GGC ATG ATG GTG GTG GCG CTT CTT TCC GCT
ACC TAT CAC ATC TGC CCC AAC CGG CTC AAC TTC CAG TTC GAC – 3′
```

(b)

```
M. sexta SID-1 like :  - - - - - - - - - ALFMLQLRRRQWNRKRAPRDEEYGIP  :  26
C. elegans SID-1    :  TNIGYTLYGAIFIVLSICR- - -RGRHEYSHVFGTY  :  516
                                A F       R     R R         G

M. sexta SID-1 like :  AHYGLLSSLGAGMMVVALLSATYHICPNRLNFQFD  :  61
C. elegans SID-1    :  ECTLLDVTIGVFMVLQSIASATYHICPSDVAFQFD  :  551
                           L     G   M       SATYHICP     FQFD
```

Figure 5.10: Newly identified *Manduca sexta* SID-1 like gene. (a) Nucleotide sequence of the newly identified transcript. **(b)** Alignment of the *M. sexta* SID-1-like deduced protein sequence with the *C. elegans* SID-1 protein sequence (NP_504372.2). Shared amino acid residues are shaded in black and the consensus sequence is included below the alignment. Numbers to the right of the alignment refer to the position in the full length *C. elegans* SID-1 protein.

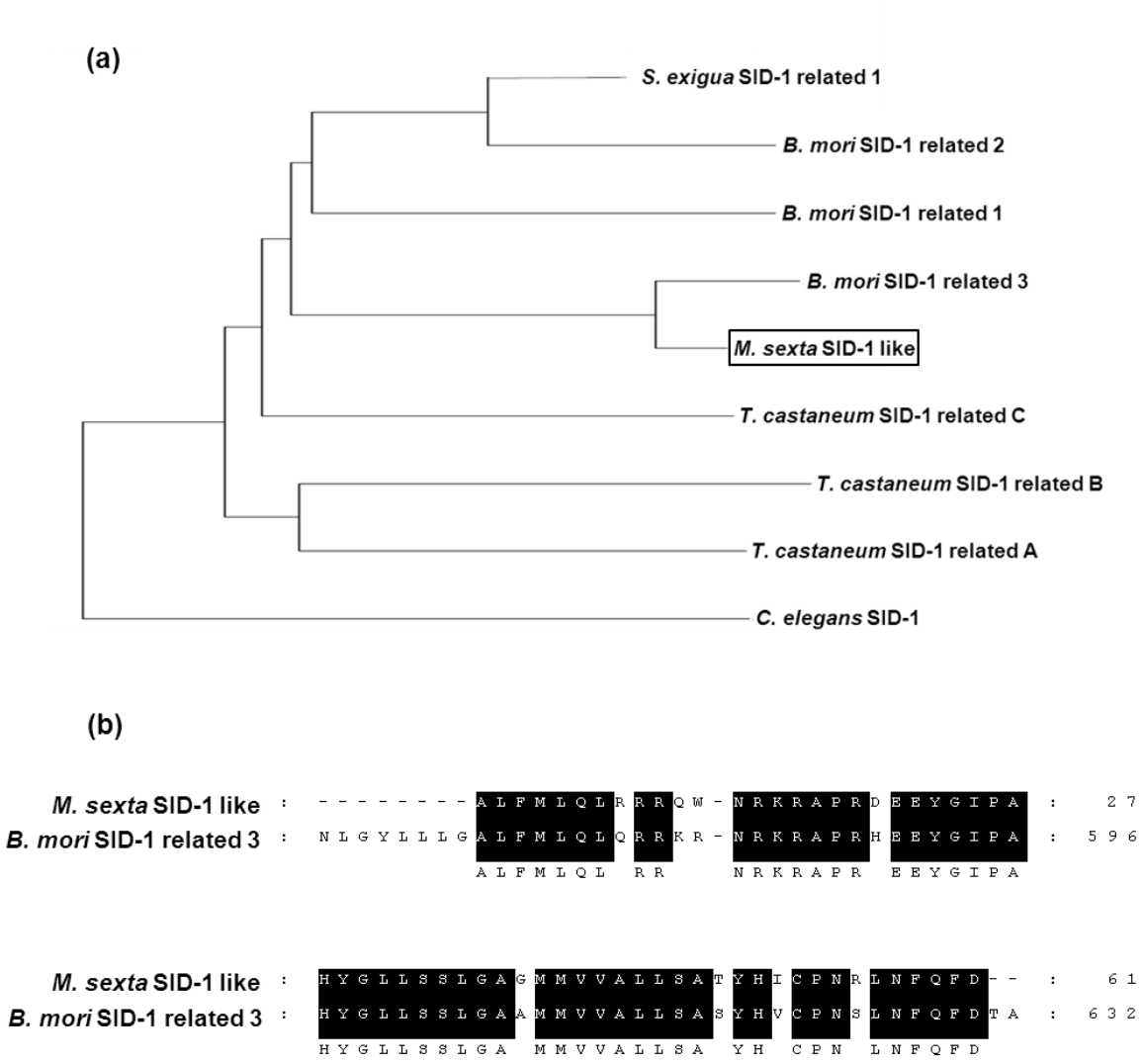


Figure 5.11: *M. sexta* SID-1-like is closely related to *B. mori* SID-1-related 3. (a) Neighbour-joining tree showing the relationship of the newly identified *Manduca sexta* SID-1-like deduced amino acid sequence with the SID-1-like genes from *S. exigua* (ACM47363.1), *B. mori* (SID-1 related 1 NP_001106735.1; SID-1 related 2 BAF95807.1; SID-1 related 3 NP_504372.2) and *T. castaneum* (SID-1 related A NP_001099012.1; SID-1 related B NP_001103253.1; SID-1 related C NP_001099128.1). The tree was rooted using the *C. elegans* SID-1 sequence (NP_504372.2) as an outgroup. **(b)** Alignment of the *M. sexta* SID-1-like deduced protein sequence with the *Bombyx mori* SID-1-related 3 protein (BAF95807.1). Shared amino acid residues are shaded in black and the consensus sequence is included below the alignment. Numbers to the right of the alignment refer to the position in the full length *Bombyx mori* SID-1-related 3 protein.

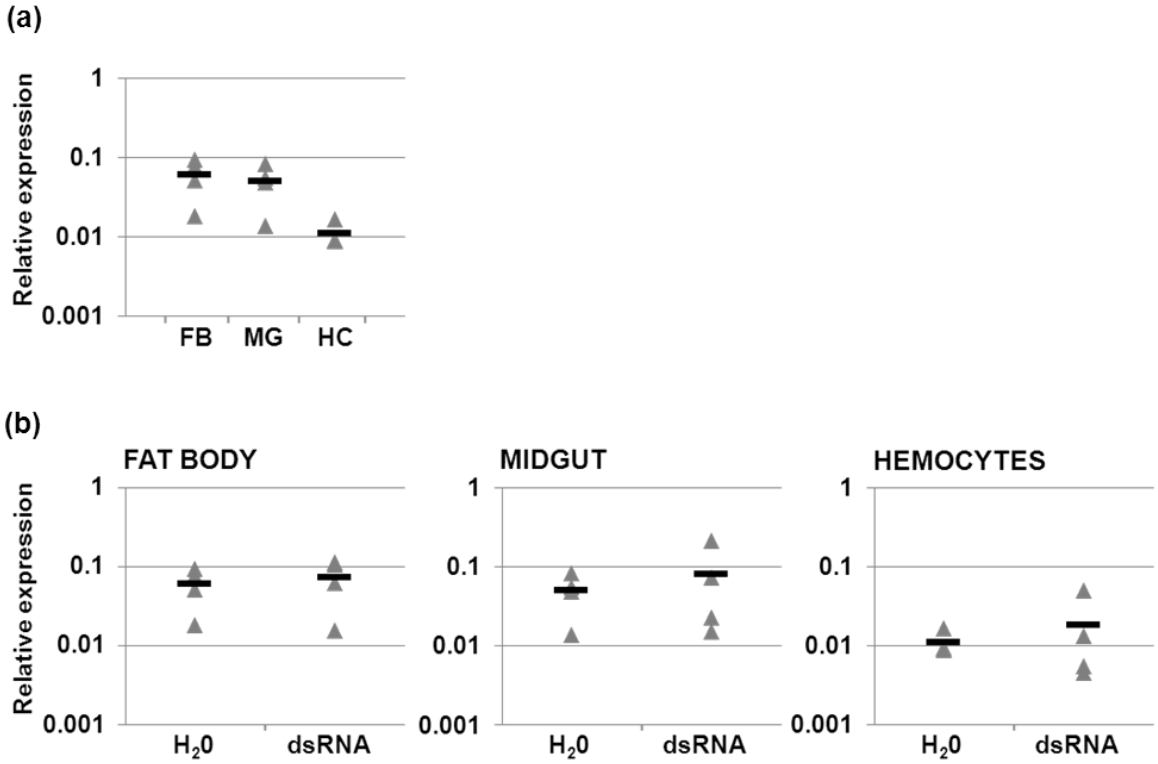


Figure 5.12: *Manduca sexta* SID-1-like expression in larval tissue. (a) Relative expression of the newly identified *Manduca sexta* SID-1 mRNA in fat body (FB), hemocyte (HC) and midgut (MG) tissue and **(b)** relative expression of SID-1 in control water-injected (H_20) and eGFP dsRNA-injected (dsRNA) insects (n=4). Newly ecdysed 5^{th} instar larvae were injected with water or 1 µg dsRNA. Six hours post injection fat body, hemocytes and midgut were dissected and subjected to q-RT-PCR to quantify mRNA levels. The values for the four replicates are shown as grey squares and the mean is depicted with a black dash.

CHAPTER 6: *DICER-2* AND *ARGONAUTE-2* ARE UPREGULATED IN RESPONSE TO DSRNA IN THE TOBACCO HORNWORM, *MANDUCA SEXTA* (LEPIDOPTERA: SPHINGIDAE).

6.1 Introduction

RNA interference (RNAi) is a general term for phenomena where double-stranded RNA molecules (dsRNAs) repress expression of homologous sequences. In exogenous (or "classical") RNAi the presence of foreign dsRNA molecules in a cell leads to the degradation of endogenous mRNA with the homologous sequence. In brief: the dsRNA molecule (or RNAi trigger) is recognised and cleaved by an RNase III-like enzyme (called Dicer-2) into shorter dsRNA molecules (21-23 nucleotides in length with 2nt long 3' overhangs and 5' phosphate and 3' hydroxyl termini), termed short interfering RNAs (siRNAs; (Zamore *et al.*, 2000; Elbashir *et al.*, 2001*a*, 2001*b*). These small ds-oligoribonucleotides are incorporated into a protein complex called RISC (RNA-induced silencing complex); one strand of the siRNA is released in an ATP-dependant manner whilst the remaining one (the guide strand) pairs with its complementary mRNA, leading to cleavage of the mRNA by a RISC endonuclease protein called Argonaute-2 (Hammond *et al.*, 2000; Nykänen *et al.*, 2001).

RNAi can be an extremely useful tool for the functional characterisation of newly identified genes, since by experimentally introducing dsRNA it is possible to knock down the expression of a specific gene in the target organism, as long as the gene sequence is known. The gene's function can then be attributed by examining the resulting phenotype. Following the revolution in sequencing technologies in the last decade and the resulting availability of large amounts of sequence data an effective method for assigning function to a gene, such as RNAi, was of paramount importance. In the field of insect biology RNAi has been used successfully as a reverse genetics tool in experiments in many insects including members of the Blattodea (Martín *et al.*, 2006), Diptera (Misquitta & Paterson, 1999; Attardo *et al.*, 2003), Coleoptera (Tomoyasu & Denell, 2004), Hemiptera (Jaubert-Possamai *et al.*, 2007), Hymenoptera (Amdam *et al.*, 2003; Lynch & Desplan, 2006), Isoptera (Zhou *et al.*, 2006), Lepidoptera (Rajagopal *et al.*, 2002), Neuroptera (Konopova & Jindra, 2008) and Orthoptera (Dong & Friedrich, 2005) and has enabled advances in many research fields from developmental biology (Martín *et al.*, 2006) and immunity (Dong *et al.*, 2006) to social behaviour (Zhou *et al.*, 2006) and pesticide resistance (Rajagopal *et al.*, 2002).

Despite the success of RNAi in many insects, it has become clear that not all insect species are equally susceptible to RNAi, with some insect species being apparently completely refractory to RNAi. There has been some speculation that particular insect species or groups of species are intrinsically less susceptible to RNAi, with a recent study addressing this problem in lepidopteran insects (Terenius *et al.*, 2011). Whilst some studies have explored the possibility that the uptake of dsRNA into insects cells may be a limiting step in RNAi experiments (Beck & Strand, 2003, 2005; Johnson *et al.*, 2010) there has, until now, been a gap in our knowledge concerning the functionality of key RNAi proteins during RNAi experiments. In fact, experiments investigating internalisation of dsRNA have indicated that processes downstream of uptake may be limiting in RNAi experiments, since some insect cell lines (e.g. *Bombyx* derived Bm5 cells) which are known to be defective in RNAi, were found to uptake dsRNA efficiently (Hannan *et al.*, 2009). In order to investigate intracellular processing of exogenous dsRNA during RNAi experiments, we have cloned partial *dicer-2* and *argonaute-2* sequences from *Manduca sexta*, an insect in which RNAi has been previously achieved (Eleftherianos *et al.*, 2009*b*), but which we have found to be variably sensitive to RNAi. We then analysed the expression of these two core RNAi genes in different tissues and in response to the injection of dsRNA. We also investigated the response of several known immune response genes to the presence of dsRNA.

6.2 Results

6.2.1 Dicer-2 *and* argonaute-2 *genes in* Manduca sexta

Two dicer-like cDNA sequences were detected (see Experimental procedures) in a *M. sexta* EST database (housed at http://insectacentral.org/). Reverse transcription-PCR using primers based on the EST data isolated 2,085 bps of dicer nucleotide sequence, which contained an open reading frame (ORF) encoding 695 amino acid residues. The ExPASy ScanProsite conserved domain search (http://expasy.org/tools/scanprosite/) predicted the putative *M. sexta* Dicer protein to encode two helicase domains and a dicer double-stranded RNA-binding fold domain, making it similar to other insect Dicer-2 proteins (but not Dicer-1 proteins) in its domain architecture (Figure 6.1a). Furthermore, the *M. sexta* dicer sequence shows high amino acid identity with conserved regions of Dicer-2 proteins from other insect species (Figure 6.1b), has amino acid identity of 65% with the recently identified *Bombyx mori* Dicer-2 protein, and falls within the Dicer-2 clade in a neighbour-joining tree of insect Dicer-1 and Dicer-2 proteins (Figure 6.1c).

An argonaute cDNA sequence was also identified in the *M. sexta* EST library. Using a 3' rapid amplification of cDNA ends PCR (RACE-PCR) strategy a 546bp clone was obtained. The sequence contained a 384 bp ORF encoding 128 aas, as well as 162 bp of 3' untranslated region. The deduced amino acid sequence was predicted by the Expasy ScanProsite search to encode a P-element-induced wimpy testis (PIWI) domain (Figure 6.2a), a domain characteristic of Argonaute proteins. Comparison with Argonaute 1, Argonaute 2 and Argonaute 3 proteins from other insect species confirmed the identity of the partially cloned *M. sexta* protein as Argonaute-2; the protein not only has 83% amino acid identity with the *Bombyx mori* Argonaute-2 protein (Figure 6.2b), but also falls within the Argonaute-2 clade in a neighbour-joining tree of insect Argonaute proteins (Figure 6.2c).

6.2.2 Expression of dicer-2 *and* argonaute-2 *in response to dsRNA*

In order to investigate the regulation of expression of core RNAi genes during an RNAi experiment, the expression of *dicer-2* and *argonaute-2* in response to injection with dsRNA (for eGFP) was determined. The amount of dsRNA injected was 1µg, which is within the range of doses of dsRNA that have been used in RNAi experiments (Terenius *et al.*, 2011). Responses were measured at 6 and 18 hours after injection with dsRNA. *Dicer-2* mRNA levels were significantly increased 6 hours after injection (relative to the control) in all three tissues tested (Figure 6.3a, t-tests on inverse transformed data: fat body $t=2.777$, $p=0.0321$; hemocytes $t=4.867$, $p=0.00280$; midgut $t=4.062$, $p=0.00664$). Although mRNA levels in dsRNA-injected insects were higher than controls at 18 hours after dsRNA injection, the difference was not statistically significant (Figure 6.3a, t-tests on inverse transformed data: fat body $t=2.068$, $p=0.0841$; hemocytes $t=2.390$, $p=0.054$; midgut $t=2.363$, $p=0.0561$). The magnitude of the increase in mRNA levels relative to the control injection at 6 hours after injection with dsRNA was substantial, being in the region of 100 fold. In contrast, at 18 hours post injection the fold increase in dicer transcript levels was always less than 10 fold.

Further experiments investigating the timing of expression in hemocytes revealed that, following injection with dsRNA, *dicer-2* mRNA expression was elevated after just 3 hours (approximately 50 x control levels), expression levels peaked at 6 hours post dsRNA injection (with a 100 fold increase in expression compared to the control), after which the expression of *dicer-2* decreased somewhat (to just 15 x and 3 x the control level 12 and 24 hours after dsRNA injection) (Figure 6.3b). *Dicer-2* mRNA levels were significantly elevated compared to the control at 3 hours ($t=5.615$, $p=0.00495$), 6 hours ($t=5.684$, $p=0.00473$), 12 hours ($t=5.329$, $p=0.00597$) and 24 hours ($t=3.478$, $p=0.0254$) post injection.

Experiments varying the dose of dsRNA injected revealed that *dicer-2* responded to dsRNA in a dose-dependent manner (Figure 6.3c). Linear regression analysis revealed that the slope of log(dose) plotted against *dicer-2* mRNA expression was significantly different from zero (F=30.863, p=9.299e-05, dicer.mRNA=0.0065(logdose)-0.00030).

The expression levels of *Manduca sexta argonaute-2* mRNA were also somewhat elevated in response to injection with dsRNA, with the mean expression 6 hours post dsRNA injection being 8, 22 & 27 times the mean control level in the fat body, hemocytes and midgut respectively (Figure 6.4a). However these changes in gene expression were non-significant (t-tests on inverse transformed data, fat body: t=-1.365, p=0.2211; hemocytes: t=-1.530, p=0.1768 & midgut: t=-1.354, p=0.2244). At 18 hours post injection there were moderate but non-significant increases in *argonaute-2* expression in dsRNA-injected insects when compared to control insects in fat body (t=-1.078 , p=0.323), hemocytes (t=-1.744 p=0.1317) and midgut (t=-0.900, p=0.419). It was noticeable that *argonaute-2* mRNA levels in all three tissues were much more variable than those of *dicer-2* (compare Figures 6.3 and 6.4).

The timing of expression of *M. sexta argonaute-2* mRNA in hemocytes in response to dsRNA was similar to that observed for *dicer-2* mRNA; *argonaute-2* mRNA levels were raised 3 hours after injection with dsRNA, expression peaked at 6 hours post injection, after which time expression decreased again (Figure 6.4b). Expression of *argonaute-2* was significantly elevated compared to the control at 3 hours (t=3.126, p=0.0353), 6 hours (t=3.250, p=0.0314) and 12 hours (t=3.079, p=0.037) post injection and there was a slight trend for elevated levels at 24 hours post dsRNA injection (t=2.392, p=0.075). The scale of the increase in *argonaute-2* expression was not comparable with the observed increase in *dicer-2* expression, with the peak expression level being only 19x the level in the control.

As with *dicer-2*, *argonaute-2* mRNA was found to respond to dsRNA in a dose-dependent manner (Figure 6.4c). Linear regression analysis showed that the slope of log(dose) plotted against *argonaute-2* mRNA expression was significantly different from zero (F=12.991, p=0.003206, argonaute.mRNA=0.083(logdose)+0.047).

6.2.3 Expression of dicer-2 *in response to other molecular triggers*

In order to determine whether the upregulation in expression of *M. sexta dicer-2* mRNA in response to double-stranded RNA is specific to dsRNA a range of other nucleic acids were injected into *M. sexta* larvae. Only single-stranded RNA and dsRNA triggered significant

increases in *dicer-2* mRNA levels (ssRNA t=-3.007, p=0.0396; dsRNA t=-3.825, p=0.01869). The response to ssRNA was about ten times less than to the same quantity of dsRNA. By contrast, injection of DNA or of Poly(I:C), a synthetic analogue of dsRNA, did not result in any significant increase in *dicer-2* mRNA expression (DNA t=1.299, p=0.2637; Poly(I:C) t=-1.910, p=0.1288).

6.2.4 Expression of dicer-2 *in response to multiple dsRNA injections*

With the aim of finding a way to prolong the observed transient upregulation of *M. sexta dicer-2* mRNA (Figure 6.3b) we were interested to determine whether multiple injections of dsRNA could induce an increase in *dicer-2* expression. An experiment was designed to compare *dicer-2* mRNA expression in hemocytes from insects injected with either i) two doses of water at t=0 and t=24 hours, ii) a dose of dsRNA at t=0 hours and a dose of water at t=24 hours, iii) a dose of water at t=0 hours and a dose of dsRNA at t=24 hours & i) two doses of dsRNA at t=0 and t=24 hours (the experimental design is outlined in Figure 6.6A). Transcript levels in hemocytes were analysed at t=0, t=6, t=24, t=30 & t=48 hours. Insects from the control treatment (two water injections) did not display an upregulation of *dicer-2* mRNA at any time following injection (Figure 6.6B), whilst insects from the single dsRNA injection treatments had elevated *dicer-2* mRNA levels 6 hours after dsRNA injection (injection at t=0, Figure 6.6C, t=4.834, p=0.002900 & injection at t=24, Figure 6.6D, t=2.231, p=0.0671). In each case, the response had largely decayed by 24 h after the dsRNA injection. When insects were injected twice with dsRNA, however, the second injection resulted in a significant elevation of *dicer-2* mRNA expression 6 hours later when compared to mRNA expression at that time point in the control treatment (Figure 6.6e, t=3.393, p=0.0146). The second response was similar in magnitude to the first.

6.2.5 Expression of immune genes in response to dsRNA

In addition to investigating the newly identified *M. sexta dicer-2* and *argonaute-2* genes, we were also interested to study transcriptional regulation in response to dsRNA in several previously characterised immune genes. The expression of four antimicrobial peptide genes (AMPs; *attacin*, *cecropin*, *gloverin* and *moricin*) and two pattern recognition proteins (PRPs; hemolin and immulectin-2) in response to dsRNA was therefore assessed in fat body, hemocyte and midgut tissue. Of the AMPs that were investigated, *attacin* mRNA did not display any considerable or significant changes in response to dsRNA injection (Figure 6.7a;

fat body F=0.0652, p=0.807; hemocytes F=0.2423, p=0.6401; midgut F=0.5347, p=0.4922). The mean mRNA expression of the remaining three AMPS (*cecropin*, *gloverin* and *moricin*) was considerably elevated in dsRNA-injected insects compared to control insects in all three tissues tested (Figure 6.7a). However, a significant increase in expression following dsRNA injection was only observed for *moricin* transcripts in fat body (F=15.639, p=0.007498) and hemocytes (F=7.0973, p=0.03732). The increased levels of *cecropin* mRNA in the fat body following dsRNA injection closely approached but did not achieve statistical significance (F=5.7555, p=0.05336), but all other increases in expression in dsRNA-treated insects were non-significant.

Expression levels of the two PRPs *hemolin* and *IML-2* did not significantly respond to dsRNA injection (Figure 6.7b). Expression was not at all elevated following dsRNA-injection in the fat body (hemolin: F=0.4231, p=0.5395 and IML-2: F=0.2193, p=0.656). Hemolin transcripts in hemocytes and midgut were more numerous in dsRNA-injected insects, but these differences in expression were non-significant (Figure 6.7b; F=1.7309, p=0.2363 and F=0.981, p=0.3602 respectively). The mean IML-2 transcript level was raised in hemocytes after dsRNA injection, but again this was non-significant (F=1.4681, p=0.2712), whereas in midgut there was a slight, but non-significant decrease in IML-2 transcript levels following dsRNA injection (F=0.981, p=0.3602).

6.3 Discussion

6.3.1 Expression of RNAi genes in response to dsRNA

In this study we were interested to test the idea that deficiency in intracellular dsRNA processing might be responsible for the apparent inefficacy of RNAi in some insects and the variable efficiency of RNAi experiments (Terenius *et al.*, 2011). We hypothesised that the expression of core RNAi genes might vary, and might be modulated in response to the presence of dsRNA. Two key components of the cellular RNAi pathway, *dicer-2* and *argonaute-2*, were therefore identified in *Manduca sexta* and their expression in response to dsRNA characterised using q-RT-PCR. These experiments revealed that *dicer-2* mRNA levels and, to a lesser extent, *argonaute-2* mRNA levels were elevated following injection with dsRNA in a specific and dose-dependent manner. This is the first study of which we are aware that concerns the expression of core RNAi genes in response to dsRNA. There is, therefore, a complete lack of comparable data in the field, but it seems likely that core RNA genes may also be upregulated in response to dsRNA in other organisms.

Our experiments were prompted by the desire to understand the well-known variability of responses in RNAi experiments. We have ourselves experienced such variability in RNAi success in *M. sexta* (unpublished). We speculate that the resting levels of expression of one or more core RNAi genes in this insect are insufficient for RNAi, and that the observed upregulation of *dicer-2* and *argonaute-2* in response to a single injection of dsRNA is not sufficiently prolonged for RNAi experiments to be consistently successful. Of course, a comparison of the expression profile in *M. sexta* with that from an insect highly susceptible to RNAi would be greatly advantageous.

6.3.2 What is the effect of multiple doses of dsRNA?

One way to potentially prolong the elevated expression of *dicer-2* and *argonaute-2* mRNA could be to inject multiple doses of dsRNA. Indeed, many successful RNAi experiments in insects have been conducted using multiple introductions of dsRNA (e.g. Araujo *et al.*, 2006; Zhou *et al.*, 2008*b*; Maestro *et al.*, 2009). Here, we investigated whether multiple injections of dsRNA resulted in multiple peaks of *dicer-2* expression and found that injecting a second dose of dsRNA does indeed cause a second peak of *dicer-2* expression. This may explain why using multiple dsRNA injections is effective in achieving an RNAi knockdown of a target gene. It may be useful to try multiple dsRNA treatments in cases where trial RNAi experiments have proved to be unsuccessful.

6.3.3 Excluding the possibility of bacterial contamination

It was important to rule out the possibility that the observed increases in core RNAi gene expression were caused by contamination of the injected dsRNA. Since the reagents used to synthesise dsRNA include recombinant proteins expressed in microbial cells, one possible type of contamination would be microbial pattern molecules, which are well known to elicit expression of immune-related genes (e.g. Eleftherianos *et al.*, 2006). We therefore quantified *dicer-2* and *argonaute-2* transcript levels in *M. sexta* larvae in response to injection with bacterial cells (*E. coli*, DH5-α). Injection with bacteria ($5x10^5$ *E. coli DH5α* cells in 50 µl PBS) did not result in elevated expression levels of *dicer-2* (relative to a control injection) in fat body at 6, 12 & 24 hours post injection (data not shown; 6 hours t=-1.905, p=0.0859; 12 hours t=-0.978, p=0.3535; 24 hours t=-0.213, p=0.83556) or in the hemocytes at 6 hours post injection (t test: t=-0.607, p=0.5606). There was also no significant increase in expression levels (relative to the control) of *argaonute-2* in bacteria-injected insects (data not shown; fat

body: 6 hours t=1.403, p=0.191; 12 hours t=-0.141, p=0.891; 24 hours t=1.563, p=0.149, hemocytes: t=-0.813 p=0.4396). Furthermore, injection with 50 µl of a 50 µg/ml solution of *Micrococcus luteus* peptidoglycan (Sigma), a bacterial pattern molecule, did not elicit a significant change in expression levels of *dicer-2* and *argonaute-2* at 6 hours post injection (data not shown; *dicer-2*; t=-0.132, p=0.8976, *argonaute-2*; t=1.625, p=0.135).

6.3.4 Immune gene upregulation following dsRNA injection

It was also of interest to study the response of previously identified *M. sexta* immune genes to injection with dsRNA. Interestingly, we found a significant increase in *moricin* mRNA expression following injection with dsRNA in fat body and hemocytes (Figure 6.7a). This finding suggests that Moricin may play a role in antiviral immunity, a proposal in direct contrast with a study reporting that expression of *moricin* was not induced by viral infection (Wang *et al.*, 2010). We also saw impressive but non-significant increases in *gloverin* expression (Figure 6.7a). Taken together with the study of Bao *et al.* (2010), which found that infection of *Bombyx mori* larvae with the *B. mori* nucleopolyhedrovirus caused a strong induction of gloverin gene expression in fat body and haemocytes, our results suggest a role for Gloverin in defence against viral injection. In addition to providing valuable information regarding the function of AMPs during viral injection, our results also make it clear that care must be taken when designing RNAi experiments to mitigate for possible induction of the target gene by the dsRNA trigger itself. The observation that expression of immune genes can be induced by dsRNA has been previously made in *Antheraea pernyi* (Hirai *et al.*, 2004).

In summary, we have identified *M. sexta dicer-2* and *argonaute-2* genes and analysed the transcriptional response of these core RNAi genes to the presence of exogenous dsRNA. It is evident that in this insect the presence of dsRNA induces expression of the RNAi machinery, suggesting that the antiviral function of RNAi may not be constitutive in this insect. Differences in the extent of basal and induced expression of core RNAi genes may therefore explain the observed differences between eukaryotic species in their susceptibility to viral infection, as well as species differences in the success of experimental RNAi procedures. Further, because prolonging the duration of contact with exogenous dsRNA has been shown to effectively prolong the period during which core RNAi gene transcripts are present at elevated levels, multiple dsRNA injections may prove to be useful in RNAi protocols.

6.4 Methods

6.4.1 Insects

Larvae of the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), were reared according to the instructions of Bell & Joachim (1976) and Reynolds *et al.* (1985). Caterpillars were kept at 25°C, 50% humidity and a photoperiod of 17h light: 7h dark. Artificial diet was prepared according to the recipe of Yamamoto (1969) as modified by Bell & Joachim (1976).

6.4.2 Molecular cloning of dicer-2 *and* argonaute-2

Several *M. sexta* expressed-sequence-tag (EST) and 454 sequencing libraries (found at insectacentral.org) were interrogated for *dicer-2* and *argonaute-2* sequences. A tBLASTn search using the *Drosophila melanogaster* Dicer-2 amino acid sequence (GI: 22026807) as a query identified two fragments of *Manduca sexta* dicer-like sequence, whilst a search using the *Drosophila melanogaster* Argonaute-2 amino acid sequence (GI: 23093414) as a query identified one fragment of argonaute-like-sequence.

To clone the dicer-like sequence RT-PCR was performed using primers based on the EST library data. Total RNA was extracted from larval fat body tissue by phenol-chloroform extraction with TRI reagent (Sigma). In order to enrich for dicer mRNA fat body tissue was dissected from a newly emerged fifth instar larva insect 6 hours after injection with dsRNA. Extracted RNA was treated with RNase-free DNaseI (Ambion) and reverse transcribed using MMLV reverse transcriptase (Promega) with random hexadeoxynucleotide primers. PCR reactions were performed using Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) under standard conditions. The primers were Dicer_F1: 5'-AGT CCC GCT GGT CAT ACA AC-3' and Dicer_R1: 5'-GAG GCA ATA GTG TCG CAT AAT CT-3' for the first round of PCR and Dicer_F2: 5'-GGC TGG TGA TGA AGC ATT TT-3 and Dicer_R2: 5'-ACG GAT CGC CCT TTA TTT CT-3' for the second, nested, round of PCR.

Argonaute was cloned by 3' RACE-PCR using a SMARTer RACE cDNA Amplification Kit (ClonTech). A nested PCR strategy using a cDNA template prepared from larval fat body was conducted as follows. The first round of PCR used a gene specific primer, based on the EST library data (Ago_gsp_F1: 5'-GGG GCA AAG GGA AGC CGG AAA T-3') and the universal primer (UP) provided in the ClonTech kit. A diluted aliquot of the resulting PCR product was used in a template in nested PCR using a downstream gene specific primer

(Ago_gsp_NeF1: 5'-CGG GGA ATC AGG CGA GGT TCA A-3') and the nested universal primer (NUP) from the kit. PCR reactions were performed using Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) and the conditions employed were heating to 95°C for 1 minute, followed by 5 cycles of 95°C for 30 seconds and 68°C for 4 minutes 30 seconds, 5 cycles of 95°C for 30 seconds, 66°C for 30 seconds and 68°C for 4 minutes and 25 cycles of 95°C for 30 seconds, 64°C for 30 seconds and 68°C for 4 minutes.

Amplified PCR products were cloned into the pCR®II-TOPO® vector (Invitrogen) and sequenced using ABI 3730xl technology.

6.4.3 Sequence analysis

Sequences were analysed using a number of different software programs. The ExPASy translate tool (found at http://expasy.org/tools/dna.html) was used to translate nucleotide sequences. Sequence alignments were performed using the CLUSTALW program (Thompson *et al*., 1994). Phylogenetic analysis was performed using the neighbour-joining method of Saitou & Nei (1987) and phylogram trees were rooted using NJplot (Perrière & Gouy, 1996). Conserved domains were identified using the ExPASy ScanProsite conserved domain search (http://expasy.org/tools/scanprosite/).

6.4.4 dsRNA synthesis

dsRNA for eGFP was synthesised by PCR and *in vitro* transcription as described in Clemens *et al.* (2000). PCR using primers with terminal 5' T7 promoter sites (eGFP_T7_F: **5'-TAA TAC GAC TCA CTA TAG GGA GA** CCT GAA GTT CAT CTG CAC CA-3' & eGFP_T7_R: **5'-TAA TAC GAC TCA CTA TAG GGA GA** GAA CTC CAG CAG GAC CAT GT-3') generated a product used as a template for *in vitro* transcription using the T7 "Megascript" kit (Ambion). Transcription was performed as per the kit instructions except that the reaction was allowed to proceed overnight. dsRNA was treated with DNase, precipitated with LiCl and resuspended in DEPC-treated water.

6.4.5 Preparation of other nucleic acids

DNA used for the injection experiment was the PCR product used as a template for dsRNA synthesis (bearing the eGFP sequence). Single-stranded RNA (ssRNA) was synthesised by

in vitro transcription. The PCR product used as the template was constructed as described above except that only one primer contained a 5' T7 sequence. Poly(I:C) was obtained from Sigma-Aldrich.

6.4.6 Injection experiments

Newly emerged 5th instar *Manduca sexta* larvae were used for all injection experiments. Insects were anaesthetised and immobilised by placing them on, and covering them in, ice for 10-15 minutes. They were surface sterilised with 70% ethanol and injected with 50 µl of 1 µg dsRNA for eGFP suspended in 50 µl DEPC-treated water or with 50 µl DEPC-treated water by puncturing the hindmost segment of the larva (anterior to the horn) with a disposable 1 mL polycarbonate 30-gauge hyperdermic needle and releasing the solution into the hemocoel. In the dose-response experiment five doses of dsRNA were injected (4µg, 400ng, 40ng, 4ng and 0.4ng). Injected larvae were incubated in the insectarium (at 25°C, 50% humidity) until the time of their dissection.

In order to dissect *M. sexta* larvae the insects were first anaesthetised, immobilised and sterilised as described above. In order to collect larval hemolymph the dorsal horn was cut at the midpoint using clean dissection scissors and bled into individual pre-chilled 1.5ml microcentrifuge tubes. To separate hemocytes from the hemolymph plasma the samples were centrifuged at 1,000 x g for 8 minutes at 4°C. To dissect tissue a cut was made directly to one side of the dorsal horn, after which the insect was opened up by cutting dorsally from the dorsal horn to just behind the head and pinned out. Fat body tissue was removed with forceps. To dissect midgut tissue a 2mm by 2mm square section of gut was cut from the dorsal side of the midgut, at the midpoint of the midgut.

6.4.7 q-RT-PCR

To conduct q-RT-PCR total RNA was first quantified with a Qubit® 2.0 Fluorometer (Invitrogen). Reverse transcription was conducted as described above. Real-time PCR was carried out using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) and iTaq SYBR Green Supermix (Bio-Rad) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye. PCR reactions were carried out in duplicate using 7.5 *p*mol specific primers and approximately 5 ng cDNA (equivalent of 5 ng RNA in RT reaction) in a total volume of 15µl. Ribosomal protein S3 (rpS-3) (GI: 527679)

was used as the internal control gene. Primer pairs were validated by standard curve analysis.

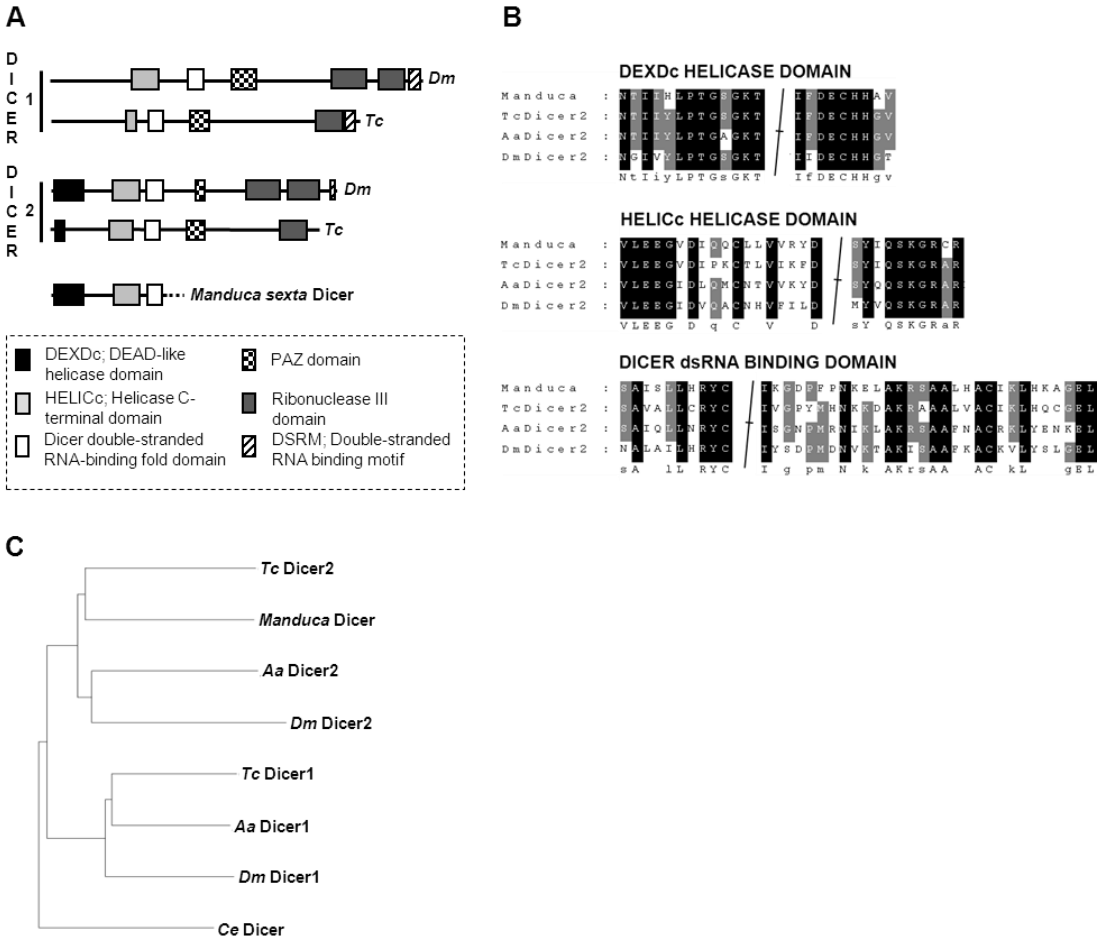
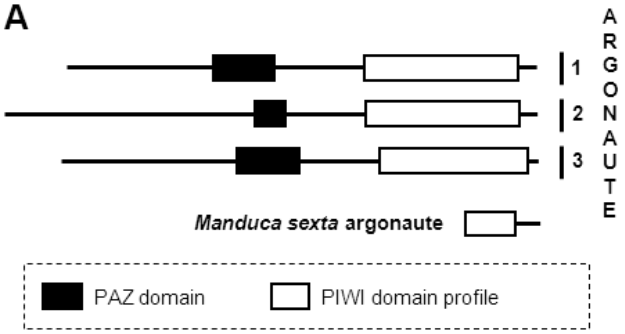


Figure 6.1: Bioinformatic analysis of the newly identified *Manduca sexta* dicer-like sequence. (A) Domain architecture of the *Drosophila melanogaster* (Dm) and *Tribolium castaneum* (Tc) Dicer-1 and Dicer-2 proteins, as well as that of the newly identified *M. sexta* Dicer sequence. (B) Highly conserved regions in a multiple alignment of *Manduca sexta* Dicer (Manduca), *Tribolium castaneum* (Tc), *Aedes aegypti* (Aa) and *Drosophila melanogaster* (Dm) Dicer-2 amino acids sequences. Where amino acid residues are shared by all four sequences they are shaded in black and where there is consensus between three of the four sequences the residues are shaded in grey. The consensus sequence is included below the alignment. (D) Neighbour-joining tree showing the relationship of the newly identified *Manduca sexta* Dicer deduced amino acid sequence with the *Drosophila melanogaster* (Dm), *Tribolium castaneum* (Tc) and *Aedes aegypti* (Aa) Dicer 1 and Dicer 2 amino acid sequences. The tree was rooted using the *C. elegans* (Ce) Dicer sequence (NP_498761.1) as an outgroup.

A



B

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Manduca :  SGNQARFNVTPGTVVDTHIVHPRELD
BmAgo2  :  PGNNARFNVDPGTVVDRDIVHPRELD
             GN ARFNV PGTVVD  IVHPRELD

Manduca :  FYLVSHQAIKGTARPTRYHAVCNDGK
BmAgo2  :  FYLVSHQAIKGTARPTRYHAVCNDGR
           FYLVSHQAIKGTARPTRYHAVCNDG

Manduca :  IPDDEVEHLTFYLCHLYSRCMRSVSY
BmAgo2  :  IPENEVEHLAYYLCHLYARCMRAVSY
           IP  EVEHL  YLCHLY RCMR VSY

Manduca :  PTPTYYAHLACMRARSLTYGQTFINH
BmAgo2  :  PAPTYYAHLACLRARSLTYGEIFNNN
           P PTYYAHLAC RARSLTYG  F N

Manduca :  DLERNPMRLRVLDRMLQHSRMFFV
BmAgo2  :  DLEKNPKRLRVLDSMLKQSRMFFV
           DLE NP RLRVLD ML  SRMFFV
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C

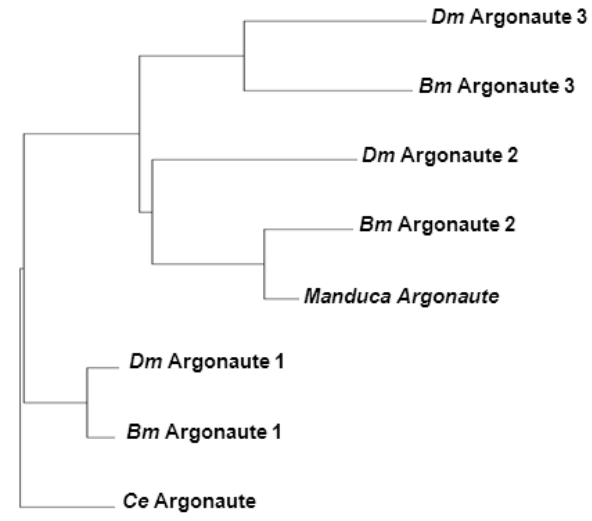


Figure 6.2: Bioinformatic analysis of the newly identified *Manduca* argonaute-like sequence. (A) Domain architecture of *Bombyx mori* Argonaute-1, Argonaute-2 & Argonaute-3 proteins as well as that of the newly identified partial *Manduca* Argonaute sequence. **(B)** Alignment of *Manduca sexta* Argonaute (Manduca) and Bombyx mori Argonaute-2 (BmAgo2) protein sequences, with conserved amino acid residues shaded in black and consensus sequence included below the alignment. **(C)** Neighbour-joining tree illustrating the relationship between the *M. sexta* Dicer deduced amino acid sequence with *Bombyx mori* (*Bm*) & *Drosophila melanogaster* (*Dm*) Argonaute 1, 2 & 3 sequences. The tree was rooted using the *C. elegans* (*Ce*) Argonaute sequence (NP_871992.1) as an outgroup.

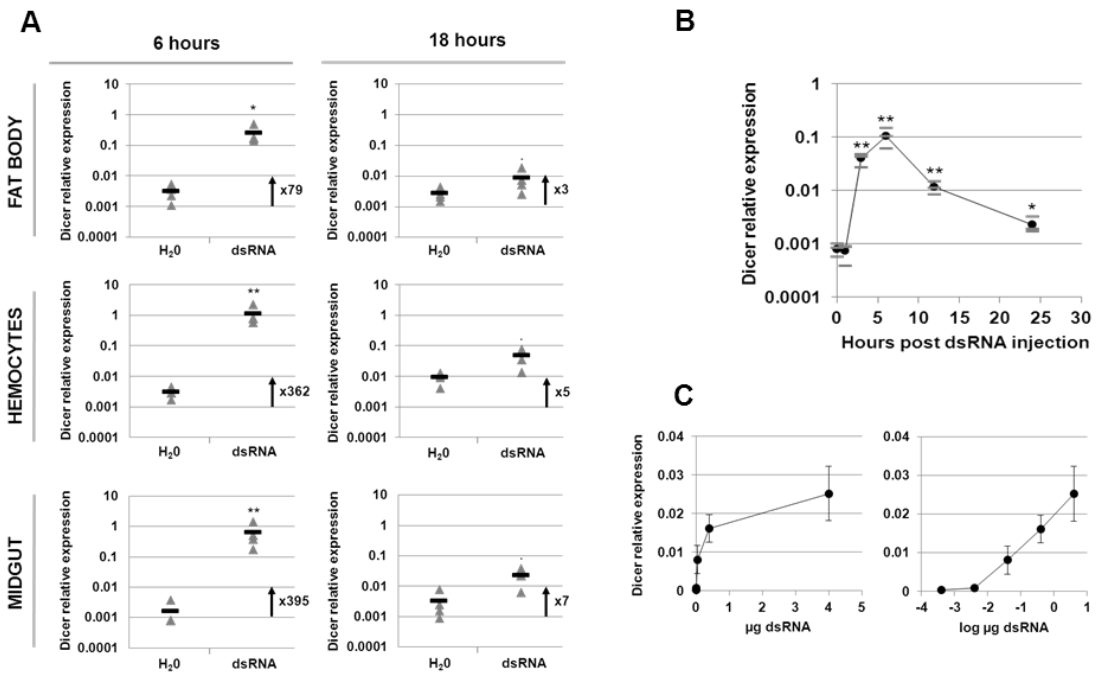


Figure 6.3: Relative expression of *Manduca sexta dicer-2* mRNA in response to dsRNA. (A) Expression of *Manduca sexta dicer-2* mRNA in control water-injected (H_20) and eGFP dsRNA-injected (dsRNA) insects. Newly ecdysed 5th instar larvae were injected with water or 1 µg dsRNA (n=4). Six hours or eighteen hours post injection fat body, hemocytes and midgut were dissected and subjected to q-RT-PCR to quantify mRNA levels. The values for the four replicates are shown as grey triangles, the mean is depicted with a black dash and the fold difference in the means is written next to a black arrow. Significant differences in transcript levels between the two treatments (water-injected and dsRNA-injected) are denoted using the following terminology for the p-values obtained from t-tests performed on the transformed data: 0.1 (.), 0.05 (*), 0.01 (**), and 0.001 (***). **(B)** Time course of *Manduca sexta* dicer-2 mRNA expression following injection with dsRNA. Newly ecdysed 5th instar larvae were injected with 1µg eGFP dsRNA and dissected for hemocytes a number of hours later (n=3). Relative mRNA levels were quantified in hemocytes using q-RT-PCR. The values for the three replicates are shown as grey dashes and the mean is depicted with a black circle. Significant differences in transcript levels from time zero are denoted using the following terminology for the p-values obtained from t-tests performed on the transformed data: 0.1 (.), 0.05 (*), 0.01 (**), and 0.001 (***). **(C)** Response of *Manduca sexta dicer-2* mRNA to different doses of dsRNA. Newly ecdysed 5th instar larvae were injected with a range of doses of eGFP dsRNA (n=4). Six hours post injection hemocytes were dissected and subjected to q-RT-PCR to quantify mRNA levels.

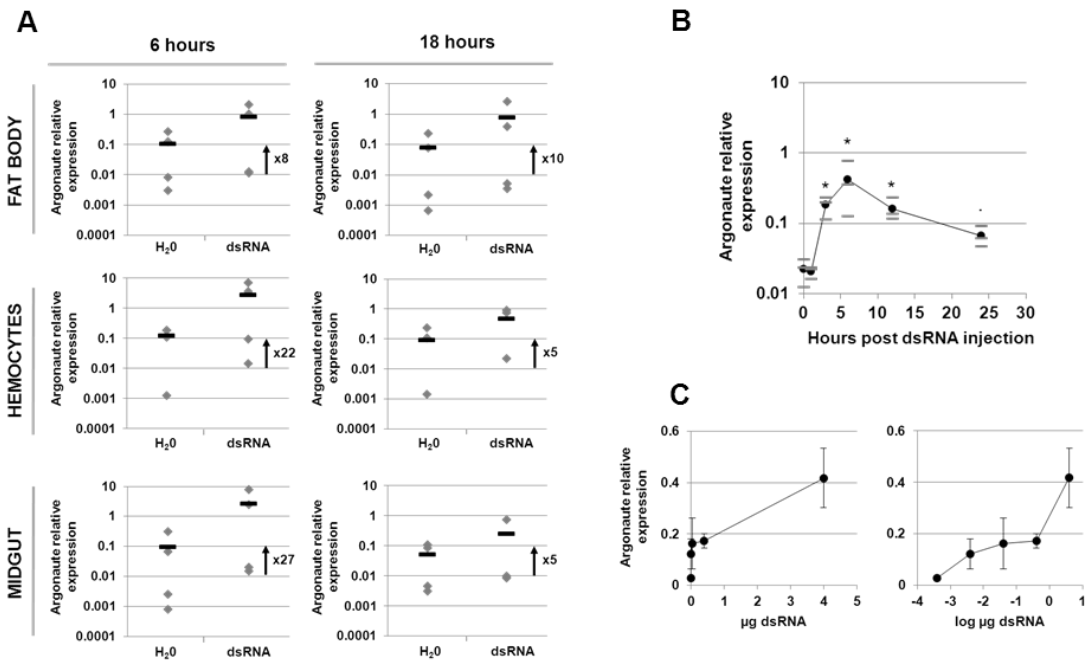


Figure 6.4: Relative expression of *Manduca sexta argonaute-2* mRNA in response to dsRNA. (A) Expression of *Manduca sexta argonaute-2* mRNA in control water-injected (H_20) and eGFP dsRNA-injected (dsRNA) insects. Newly ecdysed 5th instar larvae were injected with water or 1 µg dsRNA (n=4). Six hours or eighteen hours post injection fat body, hemocytes and midgut were dissected and subjected to q-RT-PCR to quantify mRNA levels. The values for the four replicates are shown as grey diamonds, the mean is depicted with a black dash and the fold difference in the means is written next to a black arrow. **(B)** Time course of *Manduca sexta argonaute-2* mRNA expression following injection with dsRNA. Newly ecdysed 5th instar larvae were injected with 1µg eGFP dsRNA and dissected for hemocytes a number of hours later (n=3). Relative mRNA levels were quantified in hemocytes using q-RT-PCR. The values for the three replicates are shown as grey dashes and the mean is depicted with a black circle. Significant differences in transcript levels from time zero are denoted using the following terminology for the p-values obtained from t-tests performed on the transformed data: 0.1 (.), 0.05 (*), 0.01 (**), and 0.001 (***). **(C)** Response of *Manduca sexta argonaute-2* mRNA to different doses of dsRNA. Newly ecdysed 5th instar larvae were injected with a range of doses of eGFP dsRNA (n=4). Six hours post injection hemocytes were dissected and subjected to q-RT-PCR to quantify mRNA levels.

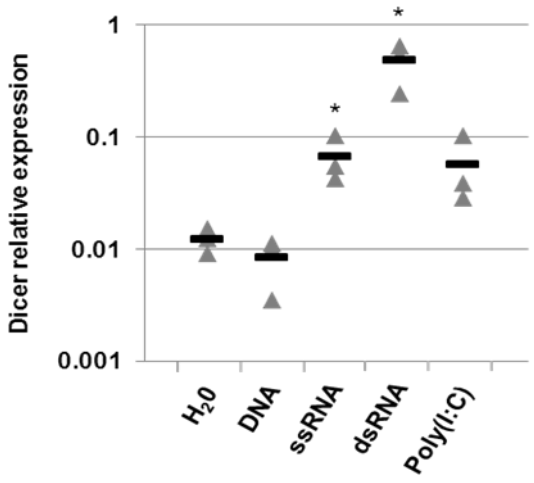


Figure 6.5: Relative expression of *Manduca sexta dicer-2* mRNA in hemocytes dissected from insects injected with H_20, DNA, ssRNA, dsRNA and poly(I:C) (n=3). Newly ecdysed 5th instar larvae were injected DEPC-treated H_20 or 1µg of nucleic acids (DNA, ssRNA, dsRNA, Poly(I:C)). Six hours post injection hemocytes were dissected and subjected to q-RT-PCR to quantify mRNA levels. The values for the three replicates are shown as grey triangles and the mean is depicted with a black dash. Significant differences in transcript levels between water-injected and nucleic acid-injected insects are denoted using the following terminology for the p-values obtained from t-tests performed on the transformed data: 0.1 (.), 0.05 (*), 0.01 (**), and 0.001 (***).

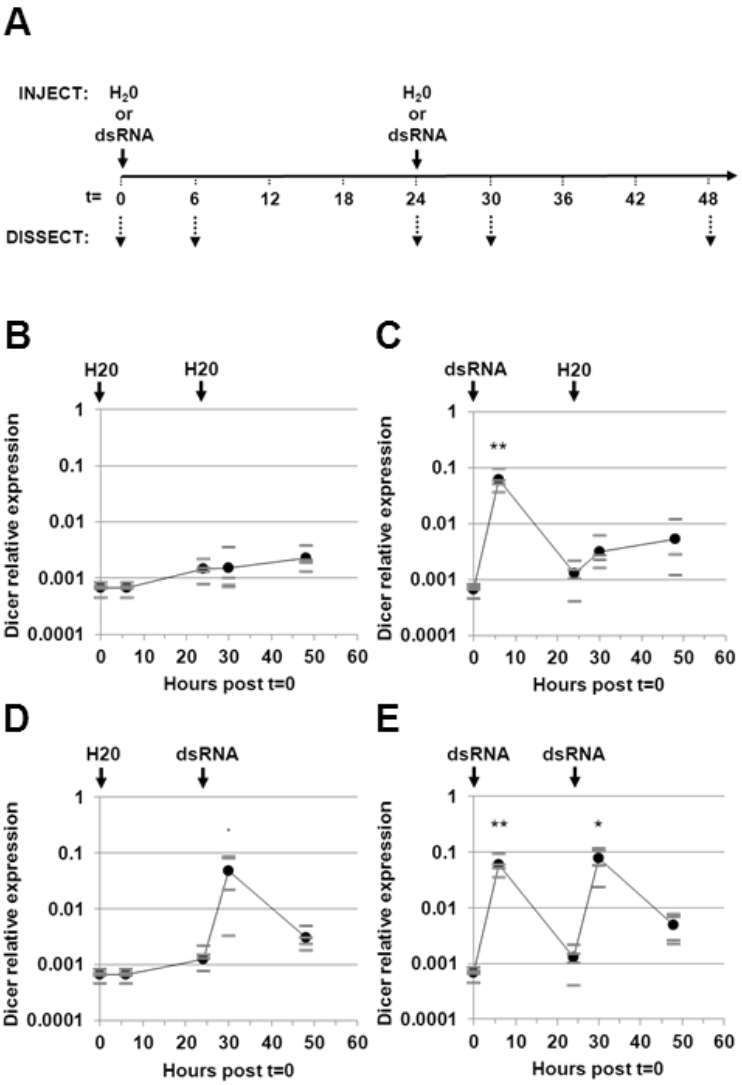


Figure 6.6: *Dicer-2* mRNA levels in response to multiple dsRNA injections. Newly ecdysed 5th instar larvae were injected with two doses of either DEPC-treated water or 1 µg dsRNA for eGFP. Hemocytes were dissected at a number of time points throughout the experiment and subjected to q-RT-PCR to quantify mRNA levels. **(A)** Design of the experiment showing times at which insects were injected with dsRNA or water and the times when insects were dissected for hemocytes. Expression of *dicer-2* mRNA in insects **(B)** injected with two doses of water, **(C)** injected with a dose of dsRNA and then a subsequent injection with water, **(D)** injected with water and then with dsRNA and **(E)** injected with two doses of dsRNA. For parts (B) to (E) the values for the four replicates are shown as grey dashes and the mean is depicted with a black circle. Significantly increased transcript levels compared to levels in control insects (twice injected with H_20) are denoted using the following terminology for the p-values obtained from t-tests performed: 0.1 (.), 0.05 (*), 0.01 (**), and 0.001 (***).

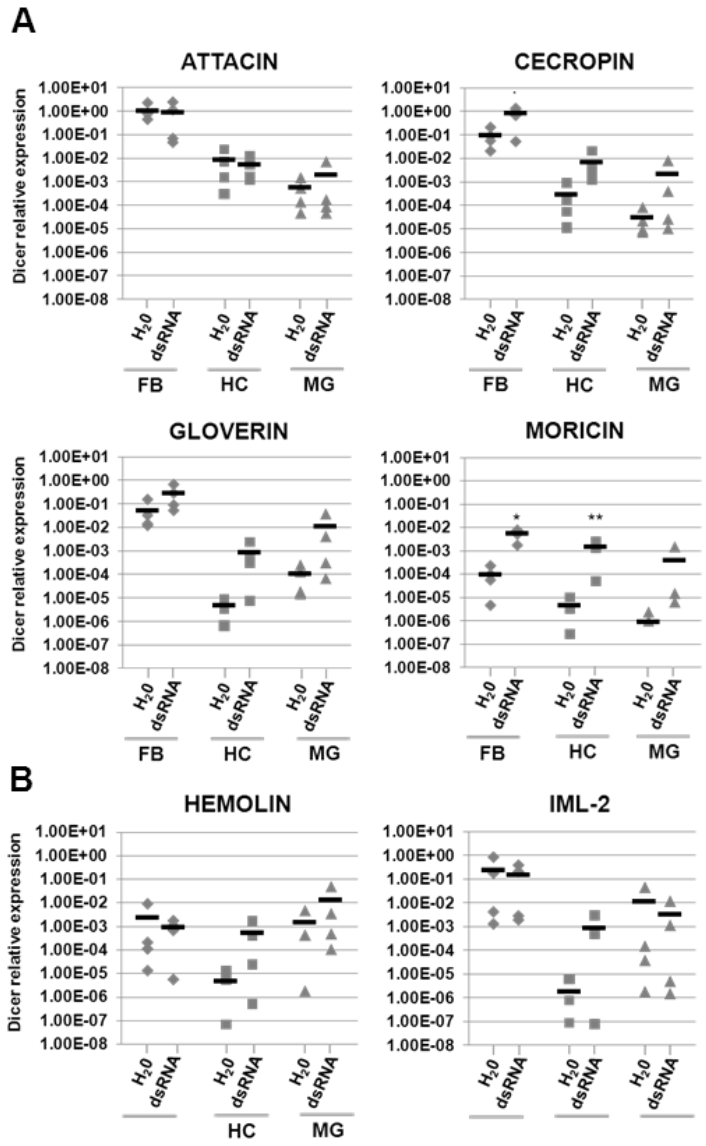


Figure 6.7: Relative mRNA expression of (A) four *Manduca sexta* antimicrobial peptides (attacin, cecropin, gloverin and moricin) and (B) two *Manduca sexta* pattern recognition proteins (hemolin and IML-2) in fat body (FB), hemocyte (HC) and midgut (MG) tissue from control water-injected (H_20) and eGFP dsRNA-injected (dsRNA) insects (n=4). Newly ecdysed 5th instar larvae were injected with water or 1 µg dsRNA. Six hours post injection fat body, hemocytes and midgut were dissected and subjected to q-RT-PCR to quantify mRNA levels. The values for the four replicates are shown as grey diamonds, squares and triangles (for fat body, midgut and hemocytes respectively) and the mean is depicted with a black dash. Significant differences in transcript levels between the two treatments (water-injected and dsRNA-injected) are denoted using the following terminology for the p-values obtained from t-tests performed: 0.05 (*), 0.01 (**), and 0.001 (***).

CHAPTER 7: DISCUSSION

RNAi is a useful reverse genetics technique that has been utilised successfully in insect species allowing advances in many different fields of insect biology (Bellés, 2010). Unfortunately, however, there is clear variability in the susceptibility of insect species to RNAi (Terenius *et al.*, 2011), a circumstance which limits the available options for analysis of gene function in less susceptible insect species. One such species is the tobacco hornworm, *Manduca sexta*, which has here been found to be, at best, variably susceptible to RNAi (see Chapter 3). In order to understand the mechanistic reason underlying the insensitivity of *M. sexta* to RNAi (and potentially shed light on the reasons for the variability in the susceptibility of other insect species to RNAi), degradation of dsRNA in hemolymph, inadequate uptake of dsRNA into tissue and the insufficient response of RNAi genes were all considered and experimentally evaluated (Chapters 4, 5 & 6 respectively).

Rapid degradation of dsRNA was found to occur in larval hemolymph of *M. sexta* (Chapter 4). However, dsRNA was found to be taken up into larval tissue (Chapter 5) and the two newly identified *M. sexta* RNAi genes, *dicer-2* and *argonaute-2*, were observed to be up-regulated in response to dsRNA injection (Chapter 6). These findings, depicted in Figure 7.1 lead to the hypothesis that degradation of dsRNA in the hemolymph may limit the success of RNAi experiments in *M. sexta.* This explanation is somewhat paradoxical, however, since there was clearly sufficient dsRNA available in the hemolymph of this insect for dsRNA to be taken up into cells (Chapter 5) and to stimulate the upregulation of RNAi genes (Chapter 6). The rapid degradation of dsRNA is, nevertheless, an interesting discovery well worth pursuing, particularly in light of the finding that the degradation of dsRNA does not occur in the hemolymph of the RNAi-sensitive German cockroach, *Blattella germanica.*

Following the discovery of enzymatic degradation of dsRNA in the hemolymph of *M. sexta,* a relatively RNAi-resistant lepidopteran insect, and the formulation of the hypothesis that breakdown of dsRNA could be responsible for insufficient RNAi in this insect, approaches with which to tackle the deficient persistence of dsRNA were considered. One strategy to enhance the efficacy of RNAi in insects like *M. sexta* would be to inhibit the breakdown of dsRNA, for instance by using RNAi itself to interfere with the nuclease responsible for the degradation. In order for this approach to be effective it would be necessary to know the identity of the enzyme responsible for dsRNA degradation. Unfortunately, however, attempts to identify the dsRNA-degrading enzyme in *M. sexta* hemolymph using a candidate approach were unsuccessful. A different course of action, which may meet with greater success, would be to purify the enzyme from the hemolymph using biochemical techniques,

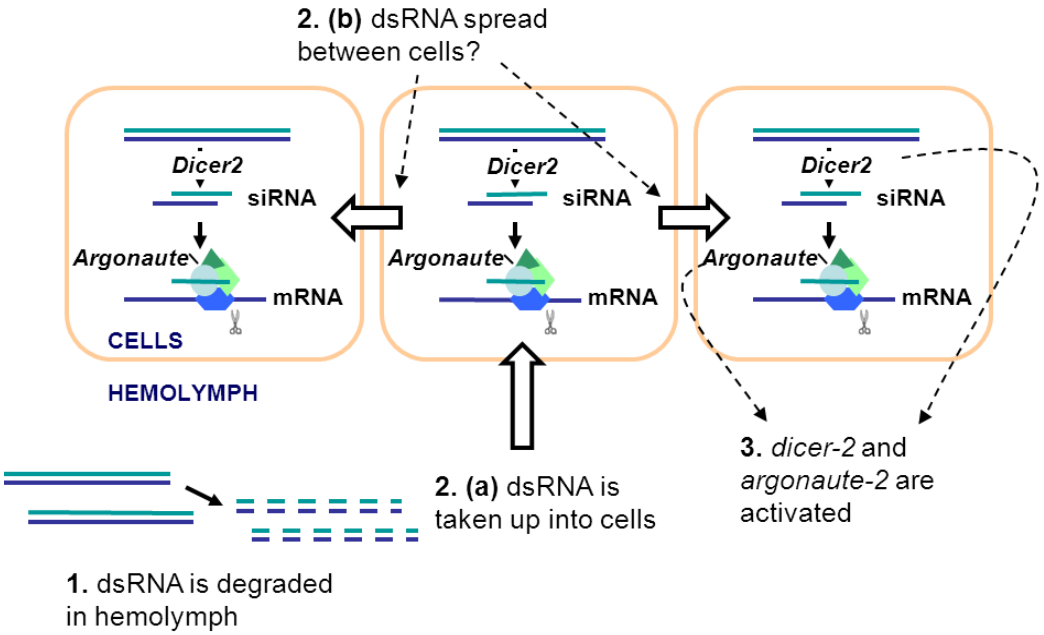


Figure 7.1: Potential causes of insensitivity to RNAi. In *M. sexta* dsRNA was would to be rapidly degraded in larval hemolymph, dsRNA was found to be taken up into larval tissue and the two newly identified *M. sexta* RNAi genes, *dicer-2* and *argonaute-2*, were observed to be up-regulated in response to dsRNA injection.

including (but not limited to) precipitation with ammonium sulphate, ion exchange chromatography and affinity chromatography (as described in Simpson, 2008). Fractions of hemolymph plasma obtained using the above methodologies could be assessed for dsRNA-specific nuclease activity using the gel-based degradation assay developed during this study (see Chapter 4) and the protein content of the fractions determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Repeated fractionation of the hemolymph plasma (using one or a combination of the above named biochemical techniques) could eventually lead to the production of a fraction retaining the ability to degrade dsRNA and only containing one protein. The sequence of the residual protein responsible for dsRNA degradation could then be resolved using, for example, MALDI-TOF mass spectrometry (Medzihradszky *et al.*, 2000).

Another approach that could be employed in order to prolong the persistence of dsRNA in the hemolymph of insects such as *M. sexta*, would be to modify the dsRNA molecules to increase their stability and reduce their susceptibility to nuclease attack or to exploit a more resilient type of dsRNA molecule as the RNAi "trigger". Whilst RNAi experiments in insects have typically involved the introduction of long dsRNA molecules, this approach was not successful in mammalian systems because mammals, unlike insects, possess an

antiviral interferon response, which acts to destroy long dsRNA molecules (Stark *et al.*, 1998). To evade this problem, researchers found that synthetic siRNA molecule could be successfully used in place of long dsRNA molecules to initiate RNAi knockdowns (Elbashir *et al.*, 2001*a*, 2001*b*). It is possible, therefore, that siRNAs could be used with equal success in insects in which large dsRNA molecules are rapidly degraded in the hemolymph. In fact, a small number of RNAi studies in insects have already made use of siRNA molecules, including several in the termite *Reticulitermes flavipes* (Zhou *et al.*, 2006, 2007; Nambu *et al.*, 2010; Schwinghammer *et al.*, 2011) as well as numerous experiments conducted with other insect species (Levin *et al.*, 2005; Mutti *et al.*, 2006; Sideri *et al.*, 2008; Chen *et al.*, 2008, 2011; Kumar *et al.*, 2009*a*; Kuadkitkan *et al.*, 2010; Huang *et al.*, 2010; Clemons *et al.*, 2011; Betanska *et al.*, 2011; Gong *et al.*, 2011).

Presumably, the researchers conducting these experiments chose to introduce siRNA molecules because they were effective in achieving reductions in transcript levels. The superior efficacy of siRNAs may be caused by their improved stability in hemolymph. However, the evidence for this is not clear, with reports of the half-life for naked siRNA in various organisms ranges from several minutes to about an hour (Layzer *et al.*, 2004; Morrissey *et al.*, 2005*a*; Dykxhoorn *et al.*, 2006; Zimmermann *et al.*, 2006).

Another approach to improve the stability of dsRNA molecules would be to chemically modify them. Various chemically modifications have been successful in improving the half-life of siRNAs, including the substitution of 2'F, 2'O-Me or 2'H residues for 2'-OH residues (Morrissey *et al.*, 2005*a*, 2005*b*), the inclusion within the molecules of phosphodiester or phosphorothioate linkages and the introduction of 2'-deoxy-2'-fluorouridine or locked nucleic acid nucleotides into the siRNAs (Braasch *et al.*, 2003). A different approach to improve stability would be to deliver dsRNA molecules in nanoparticles: dsRNAs encased in polymeric chitosan (deacetylated chitin) have been used to stimulate effective RNAi knockdowns in mammalian cells and mosquito larvae (Howard *et al.*, 2006; Zhang *et al.*, 2010) and Zhang *et al.* (2010) proposed that nanoparticles may act to stabilise the dsRNA. Introducing chemically modified synthetic dsRNAs, therefore, represents a viable possibility for improving the stability of dsRNAs in hemolymph and potentially improving the efficacy of RNAi knockdowns.

Interestingly, in contrast to the rapid degradation of dsRNA observed in *M. sexta* hemolymph plasma, no degradation of dsRNA was observed in the hemolymph of the German cockroach, *Blattella germanica*, an insect which is sensitive to RNAi (Chapter 3, Figure 3.1). In endeavouring to explain this difference it was hypothesised that the rapid degradation of dsRNA in hemolymph could represent an evolutionary response to a heavy

viral load. In other words, historic exposure of *M. sexta* to viral infection may have led to the adaptation of a constitutively active nuclease in the hemolymph, in order to suppress viral particles (which typically have a dsRNA stage in their replication cycle; DeWitte-Orr & Mossman, 2010). The presence of high levels of dsRNA-specific nuclease activity in hemolymph could limit the success of RNAi experiments, if the introduced dsRNA was degraded before it was able to be taken up into cells. Circumstantial evidence that supports this hypothesis is that lepidopterans, unlike members of the Blattodea, are known to be infected with numerous viruses (see Chapter 4).

To follow up these initial results and explore whether historic exposure to a heavy viral can lead to reduced RNAi sensitivity because of raised dsRNA-degrading activity in the hemolymph, I propose the following study. Insect species representing a wide range of taxa should be selected for experimental analysis. The viral load of the chosen group of insects should be determined (see below for how this would be done) and the susceptibility of the insects to RNAi and the persistence of dsRNA in their hemolymph experimentally ascertained (Figure 7.2). This would allow a link between viral load, persistence of dsRNA and RNAi to be explored and would potentially provide evidence for the hypothesis that insects with higher viral loads are less susceptible to RNAi, and that decreased susceptibility is linked to rapid degradation of dsRNA. Insects selected for study should be experimentally tractable and are likely to be model (or heavily studied) organisms due to the necessity for sequence data (in order to synthesise dsRNA triggers and PCR primers to assess RNAi knockdown success). Careful consideration would have to be given as to the appropriate developmental stage at which to assay viral load, efficacy of RNAi knockdowns and persistence of dsRNA. This would not be a simple matter, particularly because the developmental stages are not equivalent in holometabolous and hemimetabolous insects. It may be necessary to conduct these experiments at several different developmental stages.

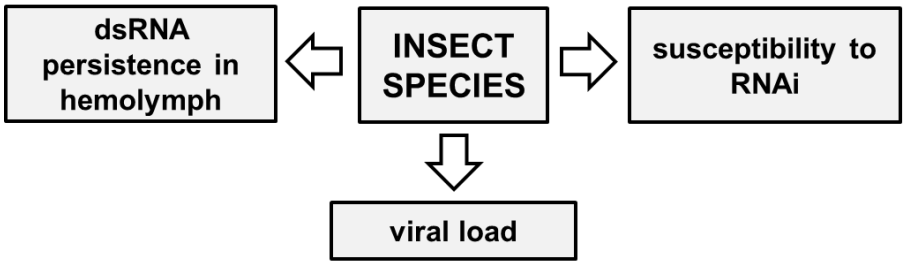


Figure 7.2: Proposed study to investigate the link between viral load, persistence of dsRNA and RNAi.

In order to make an assessment of viral load in the chosen species, deep sequencing could be employed. This technique takes advantage of the immune response of invertebrates, which processes replicating viral genomes into siRNAs of discrete sizes (Ding & Voinnet, 2007; Mlotshwa *et al.*, 2008). Wu *et al.* (2010) found that the sequences of small viral RNAs produced by the invertebrate immune system are overlapping: following the sequencing of small RNA libraries by next-generation platforms they were therefore able to assemble small RNAs into contiguous fragments. Invading viral genomes can thus be discovered in invertebrates by deep sequencing and assembly of total small RNAs. This procedure could be used to determine the number and type of viruses replicating in the insects selected for analysis.

To make an accurate appraisal of the sensitivity of the experimental insects to RNAi a set of standardised RNAi experiments should be conducted targeting a number of genes expressed in the insects (and for which there is sequence data available). It would be necessary to standardise the experimental parameters, including the dose of dsRNA introduced as the trigger (per weight of the recipient insect), the synthesis and purification methodologies used in the preparation of the dsRNA molecules, the region of the genes targeted with the dsRNA triggers, the introduction technique and the incubation time between introduction of dsRNA and determination of knockdown success. Assessment of the efficacy of the knockdowns could be determined in a precise manner using q-RT-PCR, which would allow the proportional reduction in transcript levels to be calculated.

The final step of this proposed study would be to determine the rate of degradation of dsRNA in the hemolymph of the insects. To this end the dsRNA persistence assays used in Chapter 4, including the *ex vivo* gel assay (Figure 4.2) and the q-RT-PCR-based detection method (Figure 4.1), could be employed. The q-RT-PCR assay could be used to quantify *in vivo* persistence by conducting regression analyses on time course data and comparing the gradients of the slopes. In addition, different techniques could be developed in order to quantitate degradation in hemolymph plasma *ex vivo*. For example, a modified version of the assay developed by Anfinsen *et al.* (1954) and used by Siwecka (1997) and Arimatsu *et al.* (2007), could be utilised. This technique involves the incubation of nucleic acid (we would use the synthetic dsRNA polymer poly cytidylic-inosinic acid potassium salt (Poly(I:C)) with the test solution (in this case hemolymph plasma) followed by the addition of an acidic solution to solubilise nucleotides resulting from degradation of the nucleic acid. Any remaining large nucleic acid molecules are removed by centrifugation and the absorbance of the supernatant measured spectrophotometrically at 260 nm. These techniques, used together, would reveal the rate of degradation of dsRNA in the hemolymph of the insect

species. I suggest that the above proposed study would allow a reasonable exploration of the effect of viral load on dsRNA persistence and RNAi efficacy. A potential limitation of this approach is that if the strength of an insect's RNAi response is determined by the need to deploy such responses against viruses, then the present strength and anti-viral efficacy of the RNAi response of that species will presumably be the result of co-evolutionary interactions between the insect and its viruses in the past (e.g. Obbard *et al.*, 2011). The present state of the RNAi response may therefore no longer reflect its current viral load. Nevertheless, it seems worth while exploring this possible link between the ecology of viral disease in insects and their susceptibility to RNAi procedures.

In addition to describing the persistence and uptake dynamics of dsRNA in insects this thesis reports the presence in the *M. sexta* transcriptome of *dicer-2* and *argonaute-2*-like genes. Expression studies revealed that these two newly identified genes were dramatically up-regulated in response to dsRNA injection. The initial aim of these experiments was to determine whether the absence of these genes in the *M. sexta* transcriptome or the inadequate upregulation of the genes in response to dsRNA was responsible for the relative insensitivity of this insect to RNAi. Given the degree of upregulation of these genes and the finding that *dicer-2* from *Blattella germanica*, an RNAi sensitive species, is upregulated to a similar extent (X. Bellés, personal communication, 2010) it seems unlikely that inadequate upregulation of the genes is responsible for the insensitivity of *M. sexta* to RNAi.

The potential functionality of these genes is not so clear. Partial *dicer-2* and *argonaute-2* sequences were cloned from *M. sexta* larvae using reverse transcription PCR (RT-PCR) and primers based on the sequence of contigs found in an expressed sequence tag (EST) library. However, attempts to obtain the full length sequences using a rapid amplification of cDNA ends (RACE) PCR approach were not successful, perhaps because of the probable long lengths of these genes (*dicer-2* and *argonaute-2* from *D. melanogaster* are 5,169 bps and 3,651 bps in length respectively). Therefore, whilst bioinformatics analysis of full length sequences could shed light upon the likely functionality of genes, it is not easy to draw conclusions from the partial *dicer-2* and *argonaute-2* sequences from *M. sexta*. In the absence of full length sequence data, a different approach to assessing the performance of these genes could be taken. The function of Dicer-2 proteins during RNAi is to cleave dsRNA into short RNA duplexes, termed short interfering siRNAs (Zamore *et al.*, 2000; Elbashir *et al.*, 2001*a*, 2001*b*). In order to determine whether *M. sexta* Dicer-2 acts to cleave long dsRNA molecules, an experimental approach could be employed, whereby the presence of siRNAs in insect tissue following the injection of dsRNA was determined using a

Northern blotting protocol involving RNA extraction, polyacrylamide gel electrophoresis and hybridisation (López-Gomollón, 2011).

Aside from their function in RNAi, the expression of *M. sexta dicer-2* and *argonaute-2* in response to dsRNA is interesting in itself. Expression of *dicer-2* and *argonaute-2* from *Manduca sexta* was found to be elevated following injection with dsRNA (Chapter 6, Figures 6.3A & 6.4A). This is, to my knowledge, the first report of the transcriptional upregulation of RNAi genes in response to dsRNA. In order to speculate as to the reason for this impressive upregulation of gene expression it makes sense to consider the biological role of exogenous RNAi, which is considered to be defence against pathogenic viruses (Ding, 2010). Transcriptional upregulation of RNAi genes may, therefore, be an important step during the immune response mounted by insects towards infecting viruses. Raised transcript and presumably protein levels of *dicer-2* and *argonaute-2* may be necessary to control viral infections if the viral titre is particularly high, or if the infecting virus is extensively replicating. Production of these proteins (as with any protein) is likely to be metabolically costly, hence the inducible nature of their expression.

It is interesting to speculate as to the mechanism responsible for initiating the expression of *dicer-2* and *argonaute-2*. In vertebrates, Toll-like receptors (TLRs; in particular TLR3, 7, 8 and 9) are known to detect viral nucleic acids in endosomal compartments (Alexopoulou *et al.*, 2001; Kumar *et al.*, 2009*b*; Yoneyama & Fujita, 2010), whilst retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) recognise viral RNA in the cytoplasm (Yoneyama *et al.*, 2004; Yoneyama & Fujita, 2010). With regards to invertebrates, however, *RIG-1*-like genes have only been identified in sea anemone (Putnam *et al.*, 2007) and sea urchin (Hibino *et al.*, 2006) genomes and to my knowledge there are no examples of *RIG-1*-like genes in insects. Likewise, homologues of vertebrate TLRs 3, 7, 8 & 9 are not present in insect genomes (whilst the insect Toll and mammalian TLR families are similar in size they exhibit functional and structural differences resulting from their independent expansion and diversification after the divergence of invertebrate and vertebrates; Imler & Zheng, 2004). If not TLR-like or RIG-1-like receptors, then which molecules could be involved in the detection of dsRNA molecules in *M. sexta* tissue and the subsequent signal transduction?

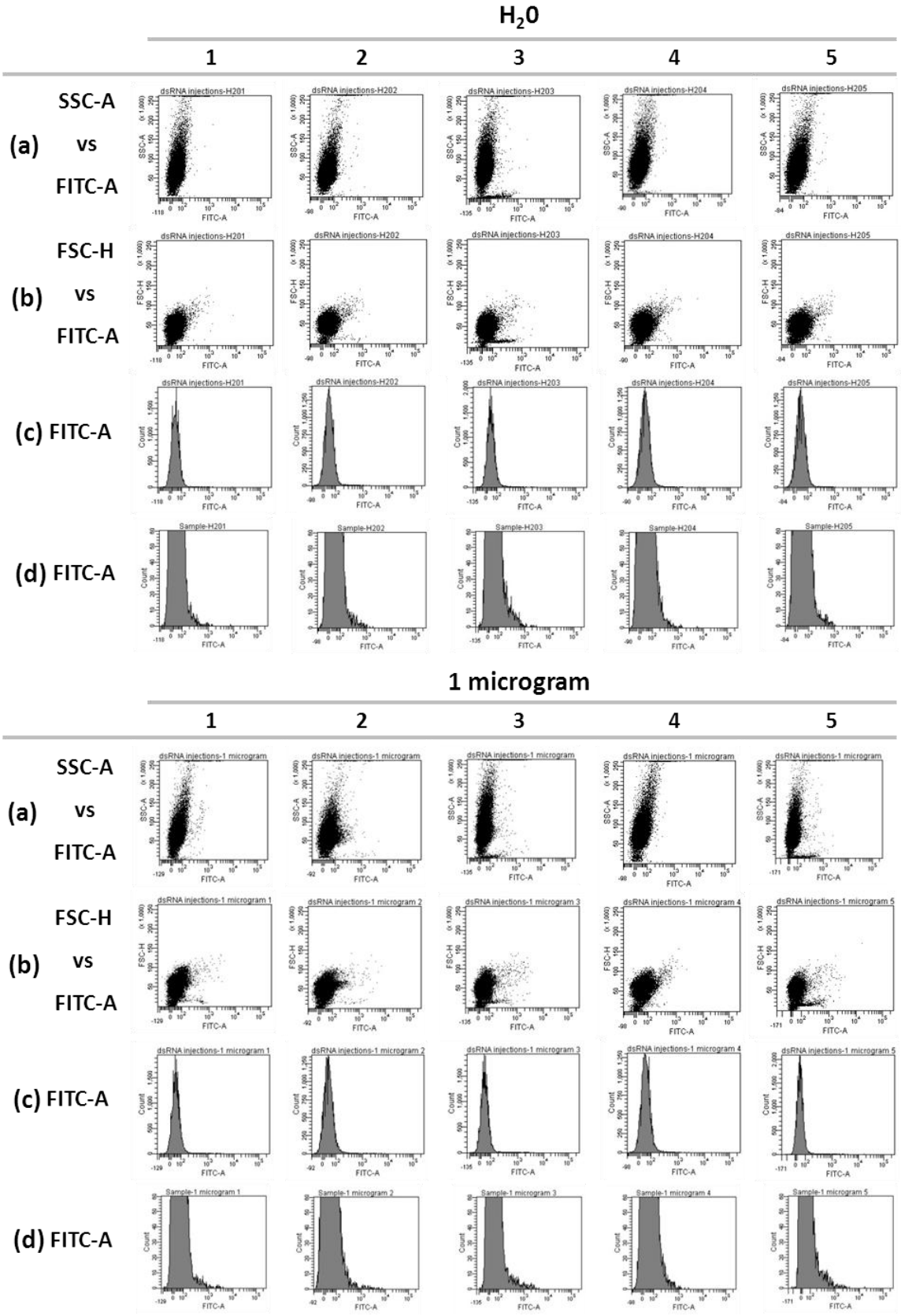
One pattern recognition receptor (PRR) candidate in insects is the immunoglobulin family protein Hemolin, which is induced in *Antheraea pernyi* larvae by synthetic dsRNA and by nuclear polyhedrosis virus (*Ap*NPV) (Hirai *et al.*, 2004). There is no direct evidence, however, for the involvement of Hemolin in dsRNA recognition. Another possibility is that Dicer-2 itself is the viral PRR, transmitting a signal which results in the induction of its own

expression, along with the expression of other effector molecules. In fact, Dicer proteins, like RIG-1-like proteins from mammals, are members of the DExD/H helicase group and, like RIG-1-like proteins, contain a classical helicase C domain and a DExD/H domain (Zou *et al.*, 2009). In addition, Dicer-2 proteins contain two dsRNA binding domains (Zou *et al.*, 2009). They are, therefore, well equipped for the recognition of dsRNA molecules. Evidence for a role of Dicer-2 as a viral PRR was provided by the study of Deddouche *et al.* (2008), which found that the induction of the *Vago D. melanogaster* gene, the product of which participates in the control of viral load, was dependent on Dicer-2. Argonaute 2 was not required for *Vago* induction, indicating that this signalling function of Dicer-2 was separate from its function in RNAi. The signalling pathway activated by Dicer-2 is unknown and Deddouche *et al.* (2008) found that the inducible expression of *Vago* did not involve the three main pathways regulating innate immunity in *Drosophila melanogaster* (Toll, Imd and Jak-STAT). There may, therefore, be an additional signalling pathway stimulated by Dicer-2-like proteins.

In this thesis I have drawn the possibly premature conclusion that, because the expression of two core RNAi genes, *dicer-2* and *argonaute-2*, responds to dsRNA, then low response of core RNAi genes after dsRNA treatment is not likely to be responsible for the insensitivity of *M. sexta* to RNAi. However, *dicer-2* and *argonaute-2* are just two of the many genes involved in exogenous RNAi in insects. The proteins R2D2, C3PO, translin and TRAX have also been found to be required in *Drosophila* for *in vivo* RNAi (Tomari & Zamore, 2005; Liu *et al.*, 2009). It is possible that one or more of these genes is deficient in its response to dsRNA. There are not currently any sequence data available for these genes in *M. sexta*. I propose that in a future study sequence data should be obtained for these genes by RT-PCR cloning (and the use of degenerate PCR primers or primers based on sequences found in EST libraries). Subsequently, q-RT-PCR could be utilised to determine the expression levels of the genes in response to dsRNA. As well as being a potential reason for the inefficient RNAi response in *M. sexta*, deficient expression of these genes may be responsible for insensitivity to RNAi in other insects. This issue has been considered by others. For example, Swevers *et al.* (2011) investigated the variable success of RNAi in lepidopteran insects by examining the expression of RNAi machinery factors in *Bombyx mori*. They found that *R2D2* was expressed at minimal levels in silkmoth tissues, whilst the silkmoth-derived Bm5 cell line was deficient in expression of *translin*. In light of this finding, it certainly would be interesting to discover whether the expression of *R2D2* and *translin* was deficient in *M. sexta* insects and whether they respond to dsRNA.

In conclusion, this study has explored several novel methods to allow the experimental examination of events occurring during RNAi experiments. Here, I report that in *M. sexta*, an insect which shows, at best, variable sensitivity to RNAi, dsRNA is rapidly degraded in insect hemolymph, that despite this degradation dsRNA is taken up into cells, and that the expression of *dicer-2* and *argonaute-2* is induced by dsRNA injection. The application of the techniques used here would allow investigators struggling to achieve successful knockdowns to determine which step is limiting in their system. For example, if uptake was found to be insufficient then they could mitigate by delivering the dsRNA along with a transfection reagents to aid uptake. Furthermore, experiments of this kind could be utilised in comparative studies to solve the problem of why insect species differ in their susceptibility to RNAi.

Appendix


H20
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(a) SSC-A vs FITC-A
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dsRNA injections-H201
dsRNA injections-H202
dsRNA injections-H203
dsRNA injections-H204
dsRNA injections-H205
Sample-H201
Sample-H202
Sample-H203
Sample-H204
Sample-H205
1 microgram
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(a) SSC-A vs FITC-A
(b) FSC-H vs FITC-A
(c) FITC-A
(d) FITC-A
dsRNA injections-1 microgram
dsRNA injections-1 microgram 1
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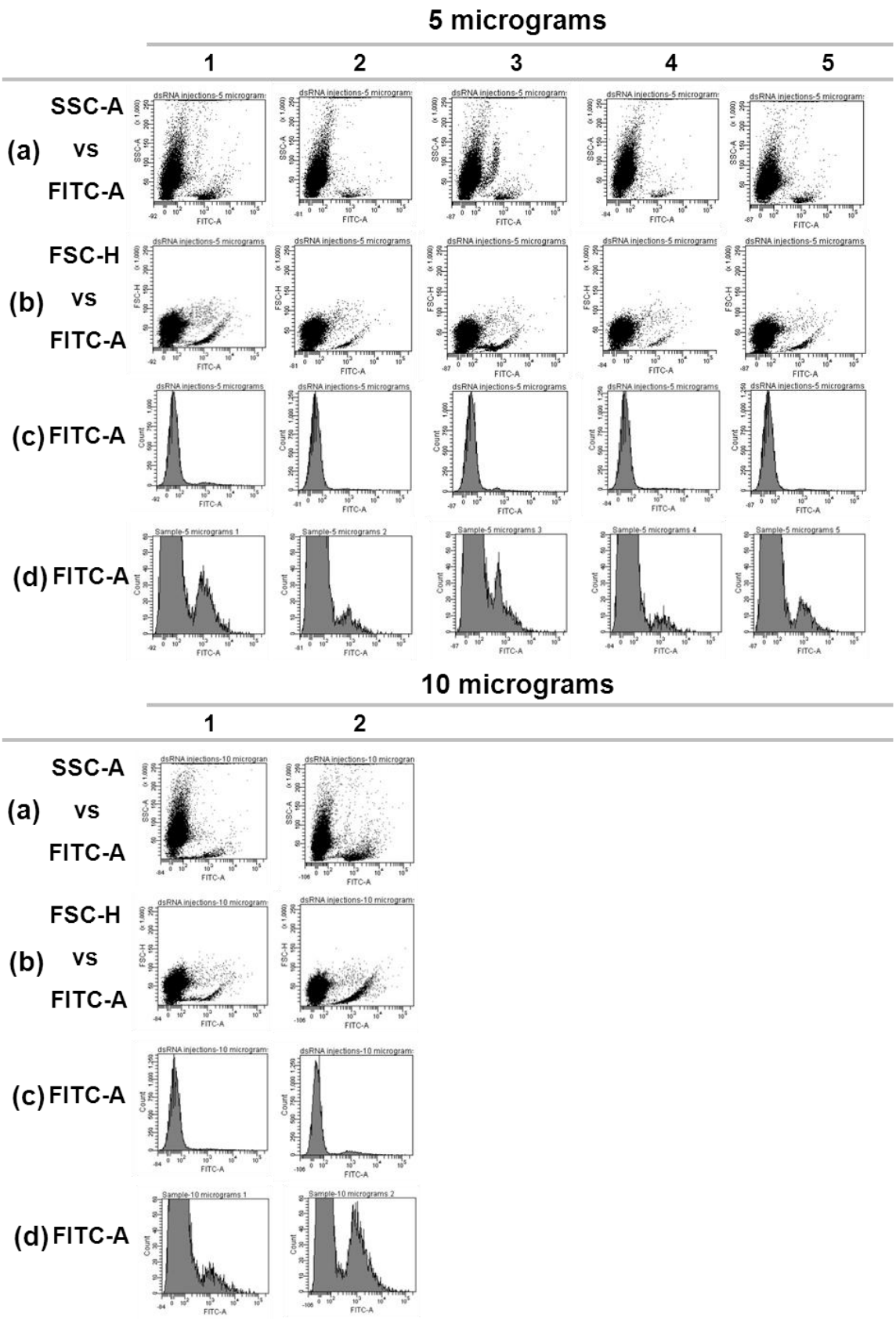
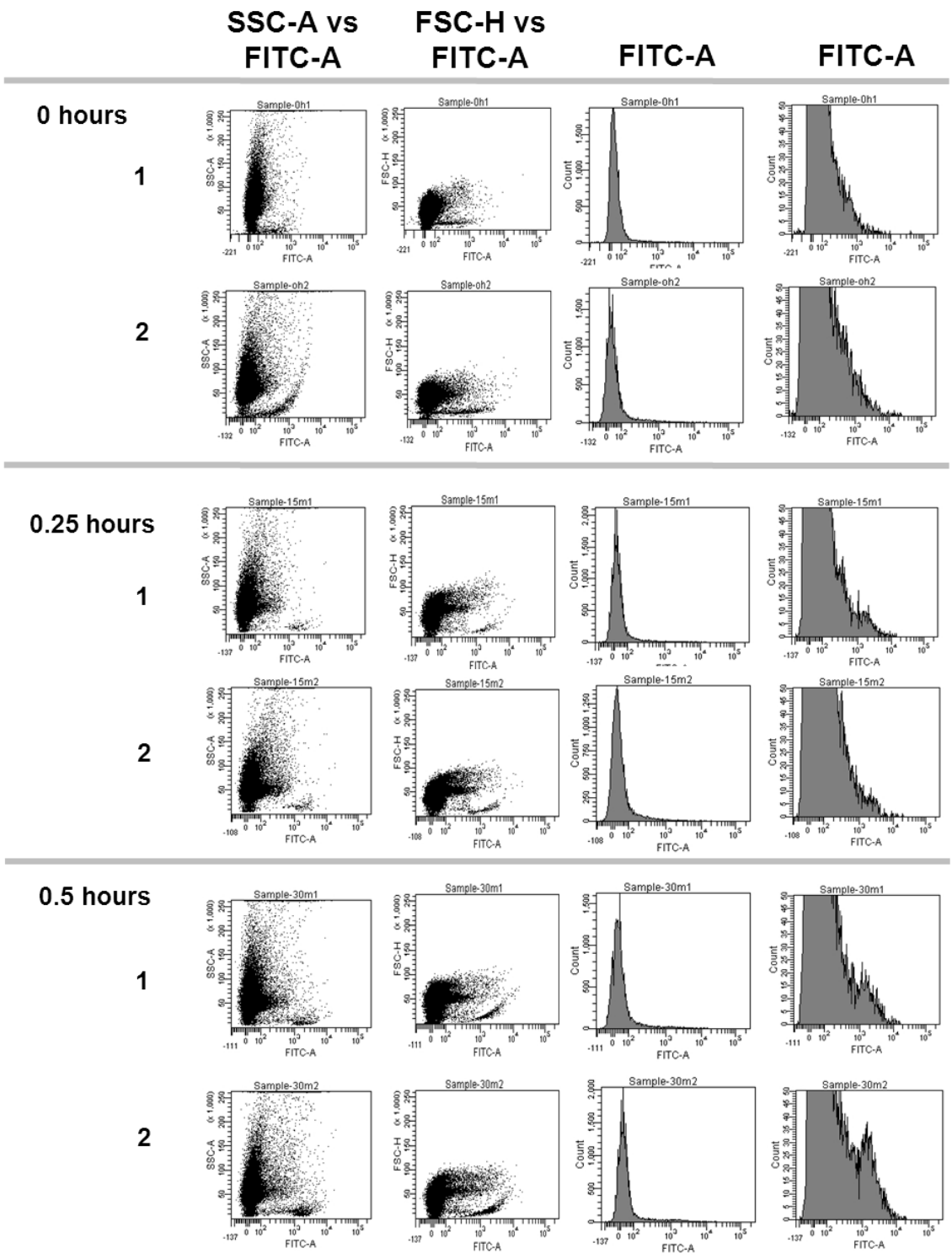

5 micrograms
1
2
3
4
5
(a) SSC-A vs FITC-A
(b) FSC-H vs FITC-A
(c) FITC-A
(d) FITC-A
10 micrograms
1
2
(a) SSC-A vs FITC-A
(b) FSC-H vs FITC-A
(c) FITC-A
(d) FITC-A

Figure A1: Uptake of dsRNA into hemocytes *in vivo*. Newly emerged 5th instar *Manduca sexta* larvae were injected with either DEPC-treated water (H_20) or different doses of fluoroscein-12-labelled dsRNA. One hour post injection hemocytes were dissected and subjected to flow cytometry analysis. **(a)** is a scatter diagram displaying 30,000 events with the side scatter of the light (SSC-A) on the y-axis and the levels of fluoroscein-12 detected (FITC-A) on the x-axis. **(b)** is a scatter diagram displaying 30,000 events with the forward scatter of the light (FSC-H) on the y-axis and the levels of fluoroscein-12 detected (FITC-A) on the x-axis. **(c)** is a histogram of FITC-A and **(d)** is a histogram of FITC-A with a reduced scale on the y-axis.


SSC-A vs FITC-A
FSC-H vs FITC-A
FITC-A
FITC-A
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Sample-0h1
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SSC-A
FSC-H
Count
FITC-A

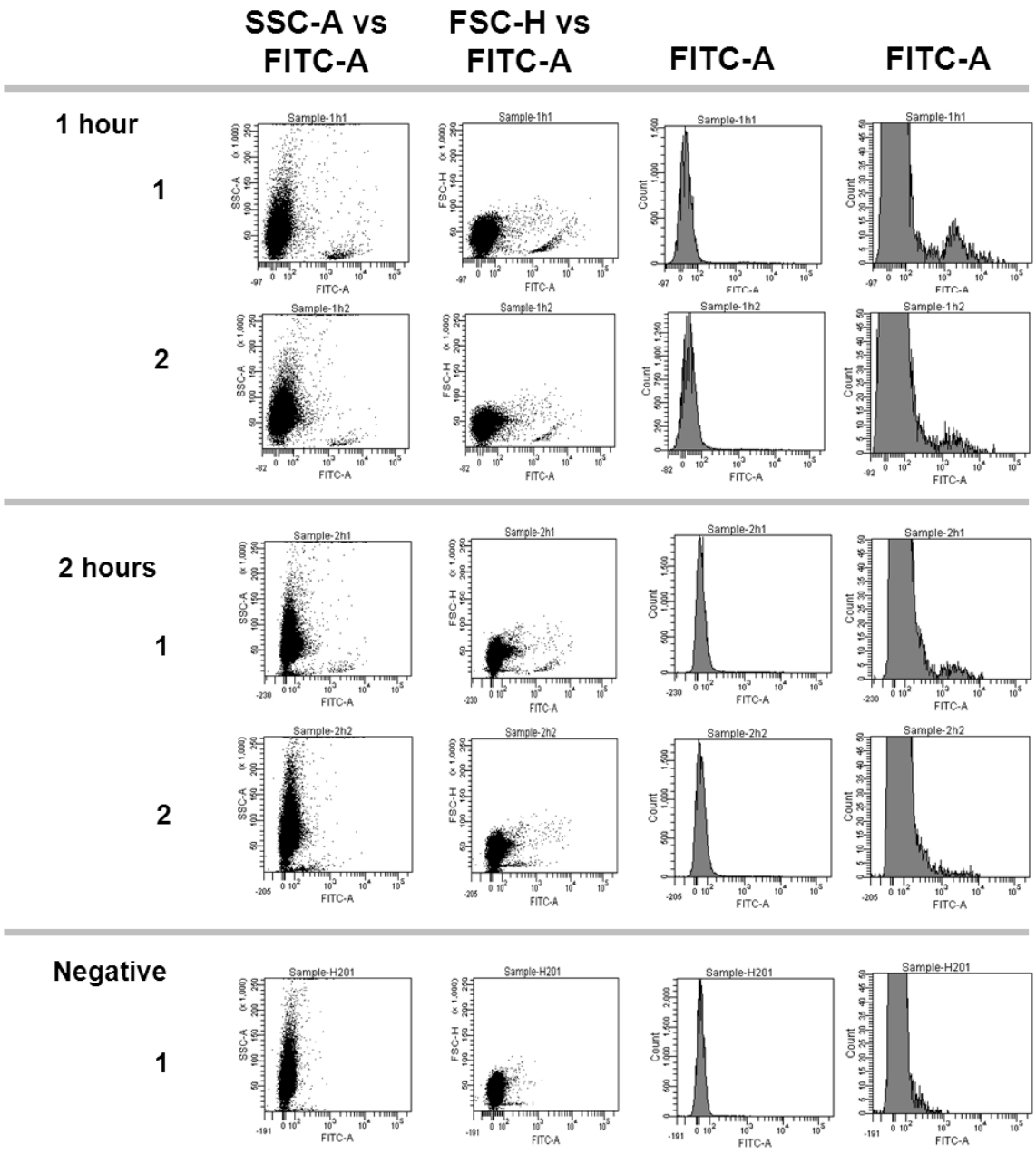

Figure A2: Time course of dsRNA uptake into cells (flow cytometry technique). Newly emerged *Manduca sexta* 5th instar larvae were injected with fluoroscein-12-labelled dsRNA and at varying intervals after injection hemocytes were dissected and subjected to flow cytometry analysis. Figures are from left to right scatter diagrams displaying 30,000 events with the side scatter of the light (SSC-A) on the y-axis and the levels of fluoroscein-12 detected (FITC-A) on the x-axis, scatter diagrams displaying 30,000 events with the forward scatter of the light (FSC-H) on the y-axis and the levels of fluoroscein-12 detected (FITC-A) on the x-axis, histograms of FITC-A and histograms of FITC-A with a reduced scale on the y-axis.

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